AF/1654

TRANSMITTAL OF APPEAL BRIEF (Large Entity)	Docket No. 112701-66

In Re Application Of: **Ballevre et al.**

Application No.	Filing Date	Examiner	Customer No.	Group Art Unit	Confirmation No.
09/508,635	May 18, 2000	D. Lukton	29157	1654	7617

Invention: **ORGAN SPECIFIC NUTRITION**

COMMISSIONER FOR PATENTS:

Transmitted herewith in triplicate is the Appeal Brief in this application, with respect to the Notice of Appeal filed on **January 9, 2006**

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Signature

Dated: **March 3, 2006**

Robert M. Barrett
Reg. No. 30,142

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant(s):	Ballevre et al.
Appl. No.:	09/508,635
Conf. No.:	7617
Filed:	May 18, 2000
Title:	ORGAN SPECIFIC NUTRITION
Art Unit:	1654
Examiner:	D. Lukton
Docket No.:	112701-066

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APPELLANTS' APPEAL BRIEF

Sir:

Appellants submit this Appeal Brief in support of the Notice of Appeal filed on January 9, 2006. This Appeal is taken from the Final Rejection in the Office Action dated November 15, 2005.

I. REAL PARTY IN INTEREST

The real party in interest for the above-identified patent application on Appeal is Nestec, Ltd. by virtue of an Assignment dated April 11, 2000 and recorded at reel 010735, frame 0634 in the United States Patent and Trademark Office.

II. RELATED APPEALS AND INTERFERENCES

Appellants' legal representative and the Assignee of the above-identified patent application do not know of any prior or pending appeals, interferences or judicial proceedings which may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

III. STATUS OF CLAIMS

Claims 30, 32, 35 and 37-41 are pending in the above-identified patent application. Claims 30, 32, 35 and 37-41 stand rejected. Therefore, Claims 30, 32, 35 and 37-41 are being appealed in this Brief. A copy of the appealed claims is included in the Claims Appendix.

IV. STATUS OF AMENDMENTS

A final Office Action was mailed on November 15, 2005. Appellants filed a Response on January 9, 2006 with an amendment to Claim 30 in reply to the final Office Action. Appellants, pursuant to the amendment, amended independent Claim 30 to delete the word "internal." This amendment was made in view of a new matter rejection posed by the Patent Office in the final Office Action. Appellants do not believe the new matter rejection was proper. However, in order to reduce the issues that need to be decided on appeal, this amendment was made.

An Advisory Action was mailed on January 30, 2006. In the Advisory Action, the amendment was considered but was deemed to raise new issues and not deemed to place the patent application in condition for allowance. Nevertheless, Appellants respectfully request that the amendment be entered in order to expedite the appeal by reducing the number of issues that need to be decided on appeal. A copy of the final Office Action and Advisory Action are attached as Exhibit A and Exhibit B, respectively, in the Evidence Appendix.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the invention by way of reference to the specification and/or figures for each of the independent claims is provided as follows:

Independent Claim 30 is directed to a method for promoting recovery of a specific internal organ of a mammal (page 2, line 30-34; page 3, line 25 to page 4, line 27; pages 14-24, Example 2), the method comprising the steps of selecting a form of a dietary milk protein hydrolysate which increases protein concentration or rate of protein synthesis in the specific organ (page 2, line 24 to page 4, line 34; page 5, line 6 to page 6, line 13); and internally administering a therapeutically effective amount of the dietary milk protein hydrolysate to the mammal (page 2, line 30 to page 4, line 34; page 9, lines 1-8).

Although specification citations are given in accordance with C.F.R. 1.192(c), these reference numerals and citations are merely examples of where support may be found in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from the specification into the claims. Pointing out specification support for the claim terminology as is done here to comply with rule 1.192(c) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the references numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description and enablement requirements.
2. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter that Appellants regard as their invention.
3. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over Nakamura (*J. Dairy Sci.* 78(6) 1253-1257, 1995) ("*Nakamura*") or Masuda (*American Institute of Nutrition* 126(12) 3063-3068, 1996) (*"Masuda"*). A copy of *Nakamura* and *Masuda* are attached herewith as Exhibits C and D, respectively.
4. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Nakamura* in view of U.S. Patent No. 5,071,867 to Ichikawa ("*Ichikawa*") or *Masuda* in view of *Ichikawa*. A copy of *Ichikawa* is attached herewith as Exhibit E.
5. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,166,132 to Gordon ("*Gordon*") or *Gordon* in view of U.S. Patent No. 6,645,942 to Verna ("*Verna*"). A copy of *Gordon* and *Verna* are attached herewith as Exhibits F and G, respectively.
6. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. WO 97/16460 to Smith ("*Smith*"). A copy of *Smith* is attached herewith as Exhibit H.
7. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 4,716,151 to Jolles ("*Jolles*"). A copy of *Jolles* is attached herewith as Exhibit I.
8. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,679,771 to Ballard ("*Ballard*") in view of U.S. Patent No. 5,661,123 to Stalker ("*Stalker*"). A copy of *Ballard* and *Stalker* are attached herewith as Exhibits J and K, respectively.
9. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over Qu, Zhensheng (*Journal of Nutrition* 126(4) 906-912, 1996) (*"Qu"*) in view of *Stalker*. A copy of *Qu* is attached herewith as Exhibit L.

10. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,723,446 to Gray ("*Gray*"). A copy of *Gray* is attached herewith as Exhibit M.

11. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Gray* in view of U.S. Patent No. 6,001,878 to Van Leeuwen ("*Van Leeuwen*") or as being unpatentable over *Gray* in view of U.S. Patent No. 5,981,590 to Panigrahi ("*Panigrahi*"). A copy of *Van Leeuwen* and *Panigrahi* are attached herewith as Exhibits N and O, respectively.

12. Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103(a) as being unpatentable over Boza, Julio (*Journal of Pediatric Gastroenterology and Nutrition* 22(2) 186-193, 1996) (*"Boza"*). A copy of *Boza* is attached herewith as Exhibit P.

VII. ARGUMENT

A. LEGAL STANDARDS

1. Written Description under 35 U.S.C. § 112, first paragraph

The first paragraph of 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention. This requirement is separate and distinct from the enablement requirement. See, e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1114 (Fed. Cir. 1991). To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. See, e.g., *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 USPQ2d 1429, 1438 (Fed. Cir. 2003); *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116.

An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.,* 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. See, e.g., *Pfaff v. Wells Elecs., Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406; *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it").

2. Enablement under 35 U.S.C. § 112, first paragraph

Any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention. The standard for determining whether the specification meets the enablement requirement is whether the experimentation needed to practice the

invention is undue or unreasonable. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Accordingly, even though the statute does not use the term "undue experimentation," it has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). See also *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) ("The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation."). A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

3. Definiteness under 35 U.S.C. § 112, second paragraph

The standard for determining whether the definitiveness requirement is met under 35 U.S.C. § 112, ¶ 2 is "whether those skilled in the art would understand what is claimed when the claim is read in light of the Specification." *Orthokinetics Inc. v. Safety Travel Chairs Inc,* 1 U.S.P.Q. 2d 1081-1088 (Fed. Cir. 1986). "If the claims, read in light of the Specification, reasonably apprise those skilled in the art both of the utilization and scope of the invention, and if the language is as precise as the subject matter permits, the Courts can demand no more." *North American Vaccine Inc. v American Cyanamid Co.,* 28 U.S.P.Q. 2d 1333, 1339 (Fed. Cir. 1993). In this regard, "[p]atent law allows the inventor to be his own lexicographer ... [T]he specification aids in ascertaining the scope and meaning of the language employed in the claims inasmuch as words must be used in the same way in both the claims and the specification. *United States v. Teletronics, Inc.,* 8 U.S.P.Q. 2d 1217, 1220 (Fed. Cir. 1988). By statute, 35 U.S.C. 112, Congress has placed no limitations on how an applicant claims his invention, so long as the specification concludes with claims which particularly point out and distinctly claim that invention." *In re Pilkington,* 162 U.S.P.Q. 145, 148 (C.C.P.A. 1996).

4. Obviousness under 35 U.S.C. § 103

The Federal Circuit has held that the legal determination of an obviousness rejection under 35 U.S.C. § 103 is:

> whether the claimed invention as a whole would have been obvious to a person of ordinary skill in the art at the time the invention was made...The foundational facts for the prima facie case of obviousness are: (1) the scope and content of the prior art; (2) the difference between the prior art and the claimed invention; and (3) the level of ordinary skill in the art...Moreover, objective indicia such as commercial success and long felt need are relevant to the determination of obviousness...Thus, each obviousness determination rests on its own facts.

In re Mayne, 41 U.S.P.Q. 2d 1451, 1453 (Fed. Cir. 1997).

In making this determination, the Patent Office has the initial burden of proving a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3d 1531, 1532, 28 U.S.P.Q. 2d 1955, 1956 (Fed. Cir. 1993). This burden may only be overcome "by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings." *In re Fine*, 837 F.2d 1071, 1074, 5 U.S.P.Q. 2d 1596, 1598 (Fed. Cir. 1988). "If the examination at the initial stage does not produce a prima facie case of unpatentability, then without more the applicant is entitled to grant of the patent." *In re Oetiker*, 24 U.S.P.Q. 2d 1443, 1444 (Fed. Cir. 1992).

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the reference or references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *In re Fine*, 837 F.2d 1071, 5, U.S.P.Q.2d 1596 (Fed. Cir. 1988). Second there must be a reasonable expectation of success. *In re Merck & Co., Inc.*, 800 F.2d 1091, 231 U.S.P.Q. 375 (Fed. Cir. 1986) Finally, all of the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 180 U.S.P.Q., 580 (CCPA 1974).

Further, the Federal Circuit has held that it is "impermissible to use the claimed invention as an instruction manual or 'template' to piece together the teachings of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 23 U.S.P.Q.2d 1780, 1784 (Fed. Cir. 1992). "One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention" *In re Fine*, 837 F.2d 1071 (Fed. Cir. 1988).

Moreover, the Federal Circuit has held that "obvious to try" is not the proper standard under 35 U.S.C. §103. *Ex parte Goldgaber*, 41 U.S.P.Q.2d 1172, 1177 (Fed. Cir. 1996). "An-obvious-to-try situation exists when a general disclosure may pique the scientist curiosity, such that further investigation might be done as a result of the disclosure, but the disclosure itself does not contain a sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued." *In re Eli Lilly and Co.*, 14 U.S.P.Q.2d 1741, 1743 (Fed. Cir. 1990).

Of course, references must be considered as a whole and those portions teaching against or away from the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve Inc.*, 796 F.2d 443 (Fed. Cir. 1986). "A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Applicant." *Monarch Knitting Machinery Corp. v. Fukuhara Industrial Trading Co., Ltd.*, 139 F.3d 1009 (Fed. Cir. 1998), quoting, *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994).

B. <u>THE CLAIMED INVENTION</u>

Independent Claim 30 is directed to a method for promoting recovery of a specific internal organ of a mammal. The method comprises selecting a form of a dietary milk protein hydrolysate which increases protein concentration or rate of protein synthesis in the specific organ. The method further comprises internally administering a therapeutically effective amount of the dietary milk protein hydrolysate to the mammal. Appellants respectfully submit that the present invention is directed, in part, to the surprisingly discovered aspect of modifying the degree of hydrolysis of a dietary protein source (e.g. selecting a form of dietary milk protein hydrolyzate), which will help determine the specific or targeted organ to be recovered. <u>In other words, by internally administering a selected protein source of a specific degree of hydrolysis, a specific organ will benefit most</u>. Teachings and examples in the specification supporting and elucidating the scope of the present invention include page 2, line 35 to page 4, line 9 and Examples 1-2.

C. CLAIMS 30, 32, 35 AND 37-41 SATISFY THE WRITTEN DESCRIPTION AND ENABLEMENT REQUIREMENTS UNDER 35 U.S.C. §112, FIRST PARAGRAPH

1. Claims 30, 32, 35 and 37-41 satisfy the written description requirement under 35 U.S.C. §112, first paragraph

a. The rejection of Claims 30, 32, 35 and 37-41 under 35 U.S.C. §112, first paragraph, with respect to the phrase "internal organ" should be reversed because the Claim 30 term "internal" was deleted in an amendment after final

In order to reduce the issues that needed to be decided on appeal, Appellants amended Claim 30 to delete the word "internal." This amendment was in view of a new matter rejection with respect to that term posed by the Patent Office in the Office Action dated November 15, 2005. The Examiner, in the subsequent Advisory Action dated January 30, 2006, refused to enter the amendment because the Appellants allegedly made no new arguments. However, Appellants respectfully submit that the amendment after final was made to return Claim 30, in part, to its original form which did not utilize the term "internal." Accordingly, to render moot the new matter rejection under 35 U.S.C. §112, first paragraph, with respect to the term "internal," Appellants agree to delete this term from Claim 30 and request that this amendment be entered.

b. The rejection of Claims 30, 32, 35 and 37-41 under 35 U.S.C. §112, first paragraph, with respect to the phrase "internally administering" should be reversed because the phrase is fully supported by the specification

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. There is no *in haec verba* requirement. Claim limitations can be supported through express, implicit or inherent disclosure.

Appellants respectfully submit that the phrase "internally administering" satisfies the written description requirement because there is sufficient support in the specification that one skilled having ordinary skill in the art would reasonably conclude that the Appellants had possession of the claimed invention. For example, support for the phrase "internally administering" can be found in the specification at page 9, lines 1-8, which describes oral nutritional forms that can be swallowed and administration of the nutritional formula via nasogastric tubes or enteral tubes, which are all aspects of internal administration. Indeed, the distinction Appellants are pointing out is between topical application and compositions that are received by the mammal internally. In this regard, it is not necessary for a term to be literally set forth in an application to be supported. Appellants have set forth how the composition can be administered enterally, through nasal gastric tubes, and by the course of eating a nutritional composition. Thus, internally administering is clearly contemplated by, disclosed in and supported by the specification.

In light of teaching of the as-filed specification and the fundamental knowledge of one having ordinary skill in the art viewing same, the skilled artisan would reasonably understand that Appellants had possession of the claimed subject matter as required by the written description requirement under 35 U.S.C. §112, first paragraph. Accordingly, Appellants respectfully submit that Claim 30 and Claims 32, 35 and 37-41 that depend from Claim 30 satisfy the requirements of 35 U.S.C. §112, first paragraph, and are in condition for allowance.

2. <u>Claims 30, 32, 35 and 37-41 satisfy the enablement requirement under 35 U.S.C. §112, first paragraph</u>

The Patent Office alleges that the specification fails to teach a skilled physiologist how to use protein hydrolysates and amino acids to promote "recovery" of an organ. Appellants respectfully disagree and submit that the specification provides adequate guidance to one of ordinary skill in the art on how to use protein hydrolysates and amino acids to promote "recovery" of an organ in accordance with the present claims.

For example, Appellants respectfully submit that one having ordinary skill in the art would readily be capable of performing the claimed method without undue experimentation. The claims are essentially directed, in part, a method comprising the steps of <u>selecting a form of</u>

a dietary milk protein hydrolysate which increases protein concentration or rate of protein synthesis in the specific organ and internally administering a therapeutically effective amount of this dietary milk protein hydrolysate to the mammal. The specification teaches various forms of a dietary milk protein hydrolysate that can be selected, for example, to increase protein concentration or rate of protein synthesis in a specific organ. Methods of internally administering these hydrolysates to mammals are not only described in the specification (e.g. orally, enterally), but are well known in the art. Indeed, the claimed method can be readily practiced without undue experimentation.

It is noted that compliance with the enablement requirement of 35 U.S.C. §112, first paragraph, does not turn on whether an example of administering the composition to the patient is disclosed. MPEP 2164.02. Nevertheless, Appellants provide sufficient working examples of the claimed invention. For example, Examples 1 and 2 show the internal administration of the hydrolyzed proteins in mammals and the corresponding increase in protein concentration and rate protein synthesis in particular organs of the mammals. Therefore, Appellants believe that the claimed invention is clearly enabled as supported by the specification.

Appellants also respectfully submit that there exists a correlation between rate of protein synthesis (e.g. hydrolysis) and promoting specific organ recovery as discovered by the Appellants. Currently, proteins, hydrolysates and free amino acids are used to meet the general needs of patients with intestinal diseases or conditions. See, specification, page 1, lines 9-19. However, these methods do not use proteins of varying degrees of hydrolysis to target specific bodily organs. Furthermore, various natural and synthetic peptides exist for targeting recovery of specific bodily organs. However, these peptides are not dietary protein and cannot serve as a primary protein source in nutritional formulas. See, specification, page 2, lines 8-20. Consequently, the advantage of the present claims is to promote recovery of specific organs using selected proteins of varying degrees of hydrolyzed, and/or free amino acids, that are dietary protein and can serve as a primary protein source.

Appellants respectfully submit that the specification does not teach or suggest that the claimed invention can treat diseases such as hepatitis, cirrhosis of the liver and kidney infection as alleged by the Patent Office. Rather, for example, the specification clearly states that feeding hydrolyzed proteins alone, or in combination with free amino acids, to patients suffering from

illnesses or damage to the <u>intestine</u> can promote recovery of the intestine. See, specification, page 8, lines 17-24.

<u>Appellants note the results showing that, depending on the degree of hydrolysis, hydrolyzed proteins and/or free amino acids promote recovery of a targeted organ, e.g. the intestine</u>. For example, the results show that Feed 4, composed of hydrolysates having degree of hydrolysis of about 35%, lead to increased protein concentration, RNA concentration, protein synthesis rate, daily protein synthesis and ribosomal efficacy in the duodenum. See, specification, page 22, lines 1-6. Additionally, though lipid and mineral contents per feed vary slightly, <u>the significant variable in the five feeds is the degree of hydrolysis</u>, where Feeds 1 and 5 are composed of intact proteins and free amino acids respectively, and Feeds 2, 3 and 4 are composed of hydrolysates with degree of hydrolysis of 14%, 17.3% and 35%. Similar to a normal, healthy person, rats with positive nitrogen balances, would generally receive whole proteins. See specification, page 1, lines 20-28. Consequently, Feed 1 of whole proteins can serve as the control. Furthermore, though the claims are drawn to promoting the recovery of organs, increased relative body weights of the tested rats, similar to rate of protein synthesis, for example, show the positive results of nutritional formulas composed of hydrolysates and/or free amino acids. See, specification, page 17, lines 5-10.

As stated above, protein hydrolysates and free amino acids can stimulate recovery of damaged organs. However, existing methods promote recovery by generally medicating rather than targeting specific organs. Appellants' present claims meet this need through results showing that, depending on the degree of protein hydrolysis, different feeds compositions target specific organs. <u>Based on these results, all a skilled artisan would need to do is (1) identify the damaged or ill organ (e.g. targeted gastrointestinal tract), (2) select the appropriate feed composition to target that specific organ based on degree of protein hydrolysis, and (3) incorporate that feed composition within a nutritional formula</u>. Numerous examples of such foodstuffs and amounts to be fed are given the specification. Consequently, one having ordinary skill in the art would be able to make or use the present claims based on the Appellants' specification without undue experimentation.

Accordingly, Appellants respectfully submit that Claim 30 and Claims 32, 35 and 37-41 that depend from Claim 30 fully comply with 35 U.S.C. §112, first paragraph, and are in condition for allowance.

D. CLAIMS 30, 32, 35 AND 37-41 ARE SUFFICIENTLY DEFINITE TO SATISFY THE REQUIREMENTS UNDER 35 U.S.C. §112, SECOND PARAGRAPH

The Patent Office alleges that, although the claims are drawn to a method of promoting "recovery" of an organ, it is unclear what the organ is recovering from. Appellants respectfully submit that, as disclosed in the specification, the claimed invention promotes the recovery (e.g. regaining or returning toward a normal or healthy state) of an organ from disease or particular condition. The claimed invention can treat, for example, premature babies with underdeveloped intestines (condition), elderly people with intestinal atrophy (condition), Crohn's disease, severe diarrhea (disease) and colitis (disease). See, specification, page 8, lines 8-28. The specification discloses recovery of organs from the examples above and diseases and conditions similar to the examples above.

In addition, the "recovery" or benefit to a specific organ can be quantitatively determined (see Example 2, the tables of pages 21-24) by measuring beneficial aspects regarding the organ such as, for example, protein concentration, RNA concentration, protein synthesis capacity, protein synthesis rate, daily protein synthesis and ribosomal efficiency as taught by the specification. These are measurable qualities and indicators of recovery. Accordingly, an increase in one or more of these measurable qualities in a specific organ can be viewed as a "recovery" of that specific organ. As a result, one having ordinary skill in the art would understand the scope of the present claims in view of the specification.

Accordingly, Appellants respectfully submit that Claim 30 and Claims 32, 35 and 37-41 that depend from Claim 30 fully comply with 35 U.S.C. §112, second paragraph, and are in condition for allowance.

E. THE REJECTIONS OF CLAIMS 30, 32, 35 AND 37-41 UNDER 35 U.S.C. §103(a) SHOULD BE REVERSED BECAUSE THE PATENT OFFICE HAS NOT ESTABLISHED A *PRIMA FACIE* CASE OF OBVIOUSNESS

1. The Cited References

Appellants respectfully submit that the obviousness rejections of Claims 30, 32, 35 and 37-41 should be reversed because the Patent Office fails to establish a *prima facie* case of obviousness. Regarding Claims 30, 32, 35 and 37-41 the Patent Office in the Final Office Action dated October 24, 2005 ("Office Action") alleges that the cited references render obvious Claims 30, 32, 35 and 37-41. However, the Patent Office fails to establish a *prima facie* case of obviousness in the rejection because there is no teaching or suggestion within the cited references cited or within the general knowledge of those skilled in the art that would have led one skilled in the art to make the combination suggested and/or the cited references fail to disclose or suggest every element of the present claims.

2. *Nakamura* and *Masuda* fail to disclose or suggest all of the elements of the claimed invention

Nakamura and *Masuda* fail to disclose or suggest all the claimed elements. Specifically, both cited references fail to disclose or suggest, as required by the claims, a method for promoting recovery of a specific organ of a mammal or selecting a form of a dietary milk protein hydrolysate (e.g. based on degree of hydrolysis) which increases protein concentration or rate of protein synthesis in a specific organ. Though both references are said to disclose using peptides from sour milk to combat hypertension, neither reference discloses any results indicating any further benefit or, specifically, any benefit to a specific organ. Moreover, the Patent Office has failed to provide any support within *Nakamura* or *Masuda* regarding same. As a result, the cited references do not even teach the problem that Appellants' invention solves.

3. <u>One having ordinary skill in the art would not be motivated to combine *Nakamura* in view of *Ichikawa* or *Masuda* in view of *Ichikawa* to arrive at the present claims</u>

Nakamura and *Masuda* are directed to using peptides from sour milk to combat hypertension. *Ichikawa* is directed to a method of healing extracellular matrix degradation to reverse glomerular sclerosis using an angiotension I converting enzyme inhibitor (ACEI). As a result, *Ichikawa* teaches a completely different compound than *Nakamura* and *Masuda* for allegedly achieving a similar objective. Nevertheless, the mechanism of each compound functions differently, and *Ichikawa* only teaches that ACEI has a therapeutic effect on kidneys. See, *Ichikawa*, column 1, lines 14-25. In this regard, *Ichikawa* does not generally represent all antihypertensive agents. Moreover, one having ordinary skill in the art would not be motivated to combine the cited references in view of their disclosures of completely different functional compounds.

To support the combination and/or modification of the cited art, the Patent Office has improperly applied hindsight reasoning by selectively piecing together teachings of each of the references in an attempt to recreate what the present claims disclose. Indeed, Appellants respectfully submit that it is only with a hindsight reconstruction of Appellants' present claims that the Patent Office is able to even attempt to piece together a rejection of the claims. As a result, the combinations of *Nakamura* in view of *Ichikawa* or *Masuda* in view of *Ichikawa* are improper and thus fail to render the claimed subject matter obvious for at least these reasons.

4. <u>*Gordon* and *Gordon* in view of *Verma* fail to disclose or suggest all of the elements of the claimed invention</u>

Gordon alone and in combination with *Verna* fails to disclose or suggest the <u>internal administering</u> of a milk protein hydrolysate as required by the present claims. Instead, *Gordon*, in attempting to heal hair and/or skin maladies, applies an enzyme modified casein <u>topically to the surface of the skin or hair</u>. See, *Gordon,* column 3, lines 4-10. *Verma* is directed to a somatic cell gene therapy method for the skin and thus fails to suggest any internal administration of a milk protein hydrolyzate. *Verma,* Abstract. Consequently, the cited references fail to disclose or suggest every element of the present claims.

Appellants also respectfully submit that one having ordinary skill in the art would not be motivated to combine a reference directed to topical use pharmaceutical composition (*Gordon*) with a reference directed to somatic cell gene therapy (*Verma*). In fact, the Patent Office does not even attempt to provide any motivation for the combination. As a result, the *Gordon* and *Gordon* in view of *Verma* fail to render the claimed subject matter obvious for at least these reasons.

5. <u>*Smith* fails to disclose or suggest all of the elements of the claimed invention</u>

Smith fails to disclose or suggest all of the claimed elements. For example, *Smith* fails to disclose or suggest a method for promoting specific organ recovery based on selecting a form of a dietary milk protein hydrolysate (e.g. selecting the degree of protein hydrolysis) which increases protein concentration or rate of protein synthesis in the specific organ as required by the present claims. The Patent Office states the same. See, Office Action, page 13, lines 1-3. Rather, *Smith* only teaches a "growth promoting activity" similar in effect to growth hormone, further exemplified by the preferred delivery mode – a food or drink product as an infant formula or animal feed. See, *Smith*, page 10, lines 14-23.

Moreover, *Smith* relates to the identification in milk of growth factors similar to IGF-1. It should be noted that such growth factors exist in milk independent of its nutritional content. This is in contrast to the present invention wherein the specific dietary milk protein hydrolysate is selected. Thus, Appellants' method of selecting a form of dietary milk protein hydrolysate, specifically required by independent Claim 30, is neither disclosed nor suggested by *Smith*.

6. <u>*Jolles* fails to disclose or suggest all of the elements of the claimed invention</u>

Jolles fails to disclose or suggest all of the claimed elements. For example, *Jolles* fails to disclose or suggest, as required by the claims, a method for promoting a <u>specific</u> organ recovery based on selecting a form of a dietary milk protein hydrolysate which increases protein concentration or rate of protein synthesis in the specific organ. Rather, *Jolles* discloses a <u>non-specific immunostimulant</u> promoting immunity against infection. See, *Jolles*, column 2, lines 5-

12. Here, "non-specific" means "a general purpose or effect," rather than a specific purpose against a specific organ. See, *Merriam-Webster's Medical Dictionary (2002).*

In addition, *Jolles* discloses the administration of a single tripeptide obtained by hydrolysis of human casein as an immunostimulant. Such a product would have a negligible nutritional content. By contrast, the present invention requires providing a therapeutically effective amount of the dietary milk protein hydrolysate to a mammal. Accordingly, *Jolles* does not disclose or suggest all of the elements of Claim 30 and dependents thereof.

7. *Ballard* in view of *Stalker* fails to disclose or suggest all of the elements of the claimed invention

Ballard in view of *Stalker* fails to disclose or suggest all the claimed elements. *Ballard* fails to disclose or suggest selecting a form of dietary milk protein hydrolysates to promote specific organ recovery as required by the claims. The Patent Office states the same. See, Office Action, page 14, lines 15-20. Likewise, *Stalker* fails to disclose or suggest the same elements. While *Stalker* is said to disclose the use of milk protein hydrolysates to treat patients with elevated protein needs, it fails to disclose targeting specific organs based on selecting a form of dietary milk protein hydrolysates for such a purpose. Accordingly, *Stalker* does not disclose altering the degree of milk protein hydrolysis to target specific organs. This is a unique aspect of Appellants' present claims and is supported in Claim 30 ("selecting a form of dietary milk protein hydrolysate which increases... rate or protein synthesis in the specific organ") and the specification. See, specification, page 5, lines 3-30. Accordingly, *Ballard* in view of *Stalker* does not disclose or suggest all of the elements of Claim 30 and dependents thereof.

8. *Qu* fails to disclose or suggest all of the elements of the claimed invention

Appellants respectfully submit that the cited references fail to disclose or suggest all the claimed elements. For example, *Qu* fails to disclose or suggest selecting a form of dietary milk protein hydrolysates to promote specific organ recovery as required by the claims. Furthermore, though *Qu* treated rats with varying concentrations of dietary protein, *Qu* fails to disclose or suggest selecting the degree of milk protein hydrolysis to target one organ versus another, which

is a unique aspect or Appellants' present claims and is supported in Claim 30 and the specification. See, specification, page 5, lines 3-30.

9. *Gray* fails to disclose or suggest all of the elements of the claimed invention

Appellants respectfully submit that the *Gray* fails to disclose or suggest all the claimed elements. *Gray* fails to disclose or suggest, as required by the claims, selecting a form of dietary milk protein hydrolysates (e.g. selecting the degree of protein hydrolysis) to promote specific organ recovery as required by the claims. Although *Gray* does disclose various embodiments with different percentages of hydrolyzed casein and whey proteins, *Gray's* experiment only tests a whole protein formula versus a single hydrolyzed protein formula. See, *Gray,* column 6, lines 22-38. Consequently, *Gray* fails to establish targeting specific organs due to varying the degrees of milk protein hydrolysis.

10. *Gray* in view of *Van Leeuwen* and *Gray* in view of *Panigrahi* fails to disclose or suggest all of the elements of the claimed invention

Appellants respectfully submit that the cited references fail to disclose or suggest all the claimed elements. As stated above, *Gray* fails to disclose or suggest selecting a form of dietary milk protein hydrolysates (e.g. selecting the degree of protein hydrolysis) to promote specific organ recovery as required by the claims. *Van Leeuwen* and *Panigrahi* fail to disclose or suggest the administration of hydrolyzed milk proteins at all. The Patent Office states the same. See, Office Action, pages 17-18. As a result, *Van Leeuwen* and *Panigrahi* also fail to disclose or suggest selecting a form of dietary milk protein hydrolysates to target specific organ recovery, as required by the present claims. Accordingly, *Gray* in view of *Van Leeuwen* or *Gray* in view of *Panigrahi* does not disclose or suggest all of the elements of Claim 30 and dependents thereof.

11. *Boza* fails to disclose or suggest all of the elements of the claimed invention

Appellants respectfully submit that *Boza* fails to disclose or suggest all the claimed elements. *Boza* fails to disclose or suggest, as required by the claims, selecting a form of dietary

milk protein hydrolysates to target specific organ recovery as required by the present claims. *Boza* concludes that intact whey proteins and hydrolyzed whey proteins are both suitable for recovery from malnutrition. See, *Boza,* page 92, column 2, lines 40-45. However, *Boza* could not make any further conclusions regarding the effectiveness of hydrolyzed whey proteins versus intact whey proteins. Furthermore, *Boza* limits its research to recovery from malnutrition, and not specific organ recovery through varying degrees or milk protein hydrolysis. As a result, *Boza* is <u>not</u> related to the growth or recovery of a specific organ and fails to disclose that a specific organ can be targeted with a milk protein hydrolysate having a pre-determined degree of hydrolysis in accordance with the present claims. This aspect, missing from *Boza*, is unique to Appellants' present claims, as supported in the claims and specification. Accordingly, *Boza* fails to disclose or suggest all of the elements of Claim 30 and dependents thereof.

12. <u>Conclusion</u>

For the reasons discussed above, the cited references, alone or in combination, do not teach, suggest, or even disclose all of the elements of Claim 30, and thus, fail to render the claimed subject matter obvious for at least these reasons. Accordingly, Appellants respectfully submit that Claim 30 and Claims 32, 35 and 37-41 that depend from Claim 30 are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

VIII. CONCLUSION

Appellants respectfully submit that Claims 30, 32, 35 and 37-41 meet the requirements of 35 U.S.C. §112, first and second paragraphs. Further, the Patent Office has failed to establish a *prima facie* case of obviousness under 35 U.S.C. §103 with respect to the rejection of Claims 30, 32, 35 and 37-41. Accordingly, Appellants respectfully submit that the written description, enablement, indefiniteness and obviousness rejections are erroneous in law and in fact and should therefore be reversed by this Board.

Respectfully submitted,

BELL, BOYD & LLOYD LLC

BY ______________________
Robert M. Barrett
Reg. No. 30,142
Customer No. 29157

Dated: March 3, 2006

CLAIMS APPENDIX

PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 09/508,635

30. A method for promoting recovery of a specific internal organ of a mammal, the method comprising the steps of:

selecting a form of a dietary milk protein hydrolysate which increases protein concentration or rate of protein synthesis in the specific organ; and

internally administering a therapeutically effective amount of the dietary milk protein hydrolysate to the mammal.

32. The method of Claim 30 wherein the specific organ is the small intestine and the dietary milk protein hydrolysate has a degree of hydrolysis of at least 30%.

35. The method of Claim 30 wherein the dietary milk protein hydrolysate comprises more than about 20% by weight of a di- and tri-peptides and a non-protein nitrogen concentration of about 60% or more of total nitrogen.

37. The method of Claim 30 wherein the mammal is suffering from muscular atrophy.

38. The method of Claim 30 wherein the mammal is suffering from a compromised gut function.

39. The method of Claim 30 wherein the dietary milk protein hydrolysate is provided in a nutritional formula.

40. The method of Claim 39 wherein the dietary milk protein hydrolysate is acceptable for premature babies having underdeveloped intestines.

41. The method of Claim 40 wherein the dietary milk protein hydrolysate comprises more than about 30% by weight of di- and tri-peptides and has a non protein nitrogen concentration of about 85% or more of total nitrogen.

EVIDENCE APPENDIX

EXHIBIT A: Office Action dated November 15, 2005

EXHIBIT B: Advisory Action dated January 30, 2006

EXHIBIT C: Nakamura (*J. Dairy Sci.* 78(6) 1253-1257, 1995) ("*Nakamura*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT D: Masuda (*American Institute of Nutrition* 126(12) 3063-3068, 1996) (*"Masuda"*), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT E U.S. Patent No. 5,071,867 to Ichikawa ("*Ichikawa*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT F: U.S. Patent No. 5,166,132 to Gordon ("*Gordon*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT G: U.S. Patent No. 6,645,942 to Verma ("*Verma*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT H: WO 97/16460 to Smith ("*Smith*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT I: U.S. Patent No. 4,716,151 to Jolles ("*Jolles*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT J: U.S. Patent No. 5,679,771 to Ballard ("*Ballard*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT K: U.S. Patent No. 5,661,123 to Stalker ("*Stalker*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT L: Qu, Zhensheng (*Journal of Nutrition* 126(4) 906-912, 1996) (*"Qu"*), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT M: U.S. Patent No. 5,723,446 to Gray ("*Gray*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT N: U.S. Patent No. 6,001,878 to Van Leeuwen ("*Van Leeuwen*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT O: U.S. Patent No. 5,981,590 to Panigrahi ("*Panigrahi*"), cited by the Examiner in the Office Action dated November 15, 2005

EXHIBIT P: Boza, Julio (*Journal of Pediatric Gastroenterology and Nutrition* 22(2) 186-193, 1996) (*"Boza"*), cited by the Examiner in the Office Action dated November 15, 2005

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EXAMINER
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References Downloaded

Please find below and/or attached an Office communication concerning this application or proceeding.

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PTO-90C (Rev. 10/03)

Office Action Summary	Application No. 09/508,635	Applicant(s) BALLEVRE ET AL.
	Examiner David Lukton	Art Unit 1654

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *8/26/05*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *30,32,35 and 37-41* is/are pending in the application.

4a) Of the above claim(s) *33 and 34* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *30,32,35 and 37-41* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☒ Notice of References Cited (PTO-892)

2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)

3)☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.

4)☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

5)☐ Notice of Informal Patent Application (PTO-152)

6)☐ Other: _____.

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 8/26/05 has been entered.

✦

Pursuant to the directives of the amendment filed 7/8/05 claim 30 has been amended. Claims 30, 32-35 and 37-41 remain pending. Claims 33-34 remain withdrawn from consideration. Applicants' arguments filed 7/8/05 have been considered and found persuasive in part.

The rejection of claims 30, 32, 35 and 37-41 as unpatentable over Tomita ('873) is withdrawn.

✦

The following is a quotation of the first paragraph of 35 U.S.C. §112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the

application was filed, had possession of the claimed invention.

Claim 30 now recites that the organ for which recovery is to be promoted has to be an "internal organ". As it happens, there is no literal support for the term "internal organ". Applicants are attempting to carve out a genus which was not described. Further, there is some ambiguity regarding the dividing line between internal organs and external ones. In applicants' opinion, are the eyes external organs, or internal ones? What about the female breast or the male genitalia? Are the ears of an elephant internal organs or external ones? And even where the skin is concerned, there is ambiguity. Certainly, the outer layers of the skin that are clearly visible would qualify as part of an external organ. But there are many layers to the skin. Where is the dividing line between skin that is part of an internal organ versus an external one? In applicants' opinion, is the stratified squamous epithelium internal or external? Is the stratum spinosum internal or external?

In addition to the foregoing, there is no description of "internal administration". It is true that on page 9, lines 6-7, the following passage is recited:

> "The nutritional formula may also be administered continuously by means of nasogastric tubes or enteral tubes…".

However, this does not provide support for "internal administration", or even parenteral administration. Nor is it clear, even at this point, what exactly is encompassed by "internal administration", or why it is that applicants believe that food which passes through the esophagus is somehow not present "internally".

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The specification fails to teach a skilled physiologist how to use protein hydrolyzates and amino acids to promote "recovery" of an organ. As stated in *Ex parte Forman* (230 USPQ 546, 1986) and *In re Wands* (8 USPQ2d 1400, Fed. Cir., 1988), the factors to consider in evaluating the need (or absence of need) for "undue experimentation" are the following: quantity of experimentation necessary, amount of direction or guidance presented, presence or absence of working examples, nature of the invention, state of the prior art, relative skill of those in that art, predictability or unpredictability of the art, and breadth of the claims.

As for the "nature of the invention", it is asserted in the specification (page 8, line 17+) that the disclosed protein hydrolyzates can be used to repair damage to the intestine. Also asserted (page 8, line 20+) is that the disclosed protein hydrolyzates can be used to treat Crohn's disease, diarrhea, colitis or sepsis, and further, that the disclosed protein hydrolyzates can be used to reverse damage to gut epithelial tissue that has resulted from a surgical procedure, or from any other cause. Though not specifically stated, the implication is that various diseases such as hepatitis, cirrhosis of the liver, and kidney infection can be successfully treated. Such diseases cause damage to organ tissue, and if the claimed

method is to be effective, the protein hydrolyzates must be effective not only to accelerate wound healing, but overcome the pathological basis of the organ damage. Applicants have argued that while the term "recovery of an organ" is intended to encompass Crohn's disease, diarrhea, colitis and sepsis, the skilled artisan reading the specification would come to believe that hepatitis, cirrhosis of the liver, and kidney infection are all excluded. However, the reasons for such a conclusion are not provided by applicants. If "recovery of an organ" can encompass treatment of Crohn's disease, diarrhea, colitis and sepsis, it stands to reason that other diseases which affect organs would be encompassed as well. Furthermore, the skilled microbiologist would expect that many organs of the septic patient would be affected, not just the bowel.

As for the "working examples", the specification discloses results which are consistent with the conclusion that if one administers a mixture of all 20 genetically encoded amino acids to a mammal, the relative weights of the stomach, intestine, duodenum jejunum, liver, gastrocnemius, soleus, and extensor will vary slightly if the ratio of amino acids is altered. This assertion is somewhat suspect, since no statistical analysis has been presented. For example, in the case of the duodenum, the standard deviation would not have to be high at all in order to justify the conclusion that the results are not statistically significant. Without further information as to the variability in the data (that is presented on page 17), it is not particularly meaningful. The results are also not meaningful, since the amount of lipids and minerals (see page 14) were varied simultaneously with the amino acid

composition. Furthermore, the total amount of amino acids varies from from feed mixture to the next. Thus, even if it turns out that the results on page 17 are statistically significant, it has not been determined the extent to which, or even whether, the observed changes in organ weights were the result of varying the amino acid composition, rather than the lipids and minerals. It may be the case that the changes in organ weights were due to changes in the total amount of amino acids administered, rather than variations in the amino acid content. Or maybe the changes in organ weights were due to changes in differential metabolism of the peptide fragments which were produced by the different hydrolysis methods (hydrolyzate 1, hydrolyzate 2 or hydrolyzate 3). Thus, in the disclosed experiments (specification) several different variables have been altered simultaneously, and it is impossible to determine the effects of any one of them taken alone. Furthermore, there is no control experiment. It has not been stated what the results are supposed to be relative to. If the feed compositions (feed 1 - feed 5) were given to rats which were already exhibiting a positive nitrogen balance, would there be any effect at all of the different feeds?

Even if it turns out that the results on page 17 are statistically significant, and if could be determined what the cause (among the numerous variables) of the variance in organ weights might be, the results are still not meaningful with respect to the claimed invention. The claimed invention is not drawn to a method of randomly altering the weights of selected organs. And even if the claims were drawn e.g., to a method of increasing the weight of the stomach, it is not at all clear how one would proceed. It may be true that if one uses,

e.g., feed #5 rather than feed #1, one will obtain a slightly higher weight of the stomach. If it were to turn out that this difference is due to the amino acid content, rather than to the lipids and minerals (or one of the other variables), it would still not be evident how one would translate the results of feed #5 versus feed #1 into a general method of increasing stomach weight. It is not apparent which amino acids are necessary, or which are sufficient; it is not made clear ~~wat~~ what degree of hydrolysis will produce the intended results, and which will not. And even if it were true that the specification taught the skilled artisan how to increase the weight of specific organs, there is no teaching as to how that teaching would translate into a showing of enablement for the claimed invention, which is that of using protein hydrolyzates and amino acids to promote "recovery" of an organ.

The results of a second experiment are presented on pages 21-24. What is shown here is that the rate of protein synthesis varies somewhat depending on which of the five feeds is used. The shortcomings of the experimental results described on page 17 apply here as well. First, the results are not statistically significant in the absence of further information as to the variability that is observed from one experiment to the next (for a given feed composition). Second, there are several different variables (with respect to the feed composition itself) which are altered simultaneously. And third, even if there were a clear assertion as to the specific variable that is supposed to correlate with the increased protein synthesis, and even if there were an experimental basis for such an assertion, this would have little relevance to the claimed invention, which is that of using protein hydrolyzates

and amino acids to promote "recovery" of an organ. The specification has presented no evidence that any such correlation exists between rate of protein synthesis, and recovery of an organ from wounding, physical trauma, or damage from an inflammatory condition. The reality is that one cannot "predict" such "recovery" based on rates of protein synthesis.

The following references discusses the issue of statistical analysis, and more importantly the issue of artifacts or invalid conclusions that can be drawn from an inadequate experimental design, or flawed assumption:

Ludbrook (*Clinical and Experimental Pharmacology and Physiology* 28 (5-6) 488-92, 2001)

Bryant (*Pediatric Allergy and Immunology* 9 (3) 108-15, 1998)

Bezeau (*Journal of Clinical and Experimental Neuropsychology* 23 (3) 399-406, 2001)

Bolton (*Journal of Clinical Pharmacology* 38 (5) 408-12, 1998)

Willenheimer (*Progress in Cardiovascular Diseases* 44 (3) 155-67, 2001)

Chung (*Plastic and Reconstructive Surgery* 109 (1) 1-6, 2002)

Atkinson (Chronobiology International 18 (6) 1041-53, 2001).

While several experiments have been conducted, there is no apparent relationship between the results of those experiments, and the claimed invention. The claimed invention encompasses repair of damage to the intestines, treatment of Crohn's disease, treatment of diarrhea, treatment of colitis or sepsis, treatment of hepatitis, treatment of cirrhosis of the

liver, and kidney infection, as well as reversal of damage to gut epithelial tissue. There is no evidence that increasing DNA synthesis or even increasing organ weight engenders a method of promoting wound healing, or of successfully treating a patient whose organs have been damaged by disease, surgery or trauma. "Undue experimentation" would be required to practice the claimed invention.

In response to the foregoing, applicants have argued that the term "recovery" is definite in scope and meaning. However, even if this is true, it does not follow therefrom that the skilled artisan can predict repair of damage to the intestines, treatment of Crohn's disease, treatment of diarrhea, treatment of colitis or sepsis, treatment of hepatitis, treatment of cirrhosis of the liver, and kidney infection, as well as reversal of damage to gut epithelial tissue on the basis of increased protein or DNA synthesis.

The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §112 second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The claims are drawn to a method of promoting "recovery" of an organ. It is unclear as to what the organ is recovering from. The term could potentially encompass recovery from a wound, physical trauma, or a disease. Despite the amendment, the line between what is encompassed and what is not encompassed remains unclear. For example, one organ is the brain. Is "recovery" from a headache encompassed, or recovery from emotional stress, or recovery from excessive alcohol consumption? It is suggested that the claim be amended to make clear what the mammal is recovering from.

The following is a quotation of 35 USC §103 which forms the basis for all obviousness rejections set forth in the Office action:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.
>
> Subject matter developed by another person, which qualifies as prior art only under subsection (f) and (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.
>
> This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103, the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made, absent any evidence to the contrary. Applicant is advised of the obligation under 37 C.F.R. 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of potential 35 U.S.C. 102(f) or (g) prior art under 35 U.S.C. 103.

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Nakamura (*J. Dairy Sci.* **78** (6) 1253-1257, 1995) or Masuda (*American Institute of Nutrition* **126**(12) 3063-3068, 1996).

As indicated previously, Nakamura discloses that peptides obtained from sour milk exhibit antihypertensive activity. Nakamura does not disclose that antihypertensive agents will promote "recovery" of a damaged heart in hypertensive patients. Masuda provides a similar teaching. Applicants have argued that neither reference discloses that antihypertensive agents are often prescribed for hypertensive patients how have suffered a

heart attack. However, this is well known in the art. The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Nakamura (*J. Dairy Sci.* **78** (6) 1253-1257, 1995) in view of Ichikawa (USP 5,071,867) or Masuda (*American Institute of Nutrition* **126**(12) 3063-3068, 1996) in view of Ichikawa ('867).

As indicated previously, Nakamura discloses that peptides obtained from sour milk exhibit antihypertensive activity. Masuda provides a similar teaching. Neither reference discloses that antihypertensive agents promote "recovery" of kidneys. Ichikawa discloses (e.g., col 1, line 21+) that antihypertensive agents promote "recovery" of kidneys. Ichikawa does not disclose administration of milk protein hydrolyzates.

Thus, the nephrologist of ordinary skill would recognize that the milk protein hydrolyzates of Nakamura and of Masuda will be effective to promote recovery of kidneys.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Gordon (USP 5,166,132).

As indicated previously, Gordon teaches that milk protein hydrolyzates can be used to treat skin, which is an organ.

Applicants have argued that the amendment to claim 30 overcomes this rejection.

However, Gordon also teaches (e.g., col 6, line 36) that the milk protein hydrolyzates can be used to treat vascular tumors or arthritis. The first point is that the medical specialist of ordinary skill would have had motivation to administer the composition subcutaneously or systemically. But in addition, there is the matter of transdermal administration. This form of administration is well known in the art. The point is that in order for the compositions of Gordon to be effective in the treatment of vascular tumors and arthritis, at least a portion of that composition would have to reach an anatomical location which would qualify as an "internal" site.

The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Gordon (USP 5,166,132) in view of Verma (USP 6,645,942). The teachings of Gordon are indicated above. Gordan does not teach that skin is an organ. Verma discloses (col 4, line 47) that skin is an organ. Verma does not disclose the use of milk protein hydrolyzates to promote recovery of an organ.

The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Smith (WO 97/16460).

As indicated previously, Smith discloses that a casein hydrolyzate has growth promoting activity. Smith does not explicitly state that the casein hydrolyzate will promote "recovery of an organ". However, one of ordinary skill would expect that growth of organs will be promoted, those of infants, as well as those of adults who have suffered damage to an organ as a result of disease, injury or surgical procedure.

In response to the foregoing, applicants have argued that Smith does not disclose "specific" organ recovery. However, given that the casein hydrolyzate has growth promoting activity, one of ordinary skill would expect that the hydrolyzate will help repair damaged organs. If at least one damaged organ is repaired, then that organ qualifies as a "specific" one.

Applicants have also argued that Smith does not disclose that the degree of absorption varies with the degree of hydrolysis. While this may be true, the claims do not require such.

The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Jolles (USP 4,716,151).

As indicated previously, Jolles discloses that tripeptides obtained from hydrolysis of milk proteins will stimulate the immune system. Jolles does not disclose that the recited tripeptides will promote recovery of an organ in an immune compromised patient.

Applicants have argued that the immunostimulatory effect is general, and one would not expect that recovery of a "specific" organ will be achieved. However, the term "specific" in the context of claim 30, is not particulary meaningful. There is not a single internal organ which is excluded by claim 30. Claim 30 encompasses the possibility that recovery of each and every internal organ can occur simultaneously, or just one of them. In the case of Jolles, one would expect that if a "specific" organ were infected with bacteria or viruses or fungi, that the tripeptides will promote recovery of that organ. As a practical matter, it is rarely the case that each and every organ becomes infected simultaneously, and to the same degree. The typical case is one in which a single organ, or perhaps two organs become infected. For such a case, recovery of the organ will be specific.

The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Ballard (USP 5,679,771) in view of Stalker (USP 5,661,123).

Ballard discloses (e.g., col 2, line 6+; col 2, line 26+; col 1, line 30+) recovery from Crohn's disease and colitis, and recovery from surgical procedures by administering IGF-1 and analogs thereof. Ballard does not disclose a method of promoting recovery by administering hydrolyzed milk proteins. Stalker discloses (col 3, line 50) administration of hydrolyzed milk proteins to patients who have "elevated protein requirements". Stalker further discloses (e.g., col 3, line 40; col 5, line 47) that persons

afflicted with Crohn's disease have "elevated protein requirements" and would benefit from the hydrolyzed milk proteins. While disclosing that persons suffering from Crohn's disease would benefit from the hydrolyzed milk proteins, Stalker stops short of asserting that the inflammation associated with the Crohn's disease will actually be mitigated.

In response to the foregoing, applicants have argued that the instant claims require recovery of a "specific" organ, as opposed to a "general" organ. However, the term "specific" in the context of claim 30, is not particulary meaningful. There is not a single internal organ which is excluded by claim 30. Claim 30 encompasses the possibility that recovery of each and every internal organ can occur simultaneously, or just one of them. Thus, for all practical purposes, the claims do not actually require that recovery occur in just one particular organ (or three organs). But as it happens, this rejection is directed especially to promoting recovery in patients who are suffering from Crohn's disease. The bowel is the organ preferred by applicants in which to promote recovery. The references taken together teach this.

Applicants have also argued that Stalker does not disclose that the degree of absorption varies with the degree of hydrolysis. While this may be true, the claims do not require such.

The rejection is maintained.

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Qu, Zhensheng (*Journal of Nutrition* 126(4) 906-912, 1996) in view of Stalker (USP 5,661,123).

As indicated previously, Qu discloses that protein malnutrition is manifest in various ways both biochemically and physiologically; one of those manifestations is suboptimal liver growth. Qu further discloses that the deficiency in liver growth which accompanies protein malnutrition can be reversed by administering proteins, such as casein; in other words, proteins promote "recovery" of the liver from protein malnutrition. Qu does not disclose that hydrolyzed milk proteins can serve as a protein source.

Applicants have argued that Stalker does not disclose that varying the degree of hydrolysis has the effect of altering the extent to which benefit accrues to one specific organ versus another. While applicants may be correct on this point, the claims are not drawn to a method of varying the degree of hydrolysis, or a Jepson claim in which the improvement is varying the degree of hydrolysis. The rejection is maintained.

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Gray (USP 5,723,446).

Gray discloses (col 2, line 57+) a method of treating patients suffering from burns, and from surgical procedures. The method calls (col 3, line 27) for administration of

hydrolyzed milk protein.

In response, applicants have argued that Gray does not disclose that varying the degree of hydrolysis has the effect of altering the extent to which benefit accrues to one specific organ versus another. While applicants may be correct on this point, the claims are not drawn to a method of varying the degree of hydrolysis, or a Jepson claim in which the improvement is varying the degree of hydrolysis. The rejection is maintained.

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Gray (USP 5,723,446) in view of Van Leeuwen (USP 6,001,878).

Gray discloses (col 2, line 57+) a method of treating patients suffering from burns, and from surgical procedures. The method calls (col 3, line 27) for administration of hydrolyzed milk protein. The reference also suggests (col 3, line 47; col 5, line 56) administration of glutamine in addition to the hydrolyzed milk protein. Gray does not disclose that glutamine will promote recovery of an organ. Van Leeuwen discloses that glutamine will promote recovery of the liver. Van Leeuwen does not disclose administration of hydrolyzed milk proteins.

In response, applicants have argued that Gray does not disclose that varying the degree of hydrolysis has the effect of altering the extent to which benefit accrues to one specific organ versus another. While applicants may be correct on this point, the claims are not drawn to a method of varying the degree of hydrolysis, or a Jepson claim in which the

improvement is varying the degree of hydrolysis. The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Gray (USP 5,723,446) in view of Panigrahi (USP 5,981,590).

Gray discloses (col 2, line 57+) a method of treating patients suffering from burns, and from surgical procedures. The method calls (col 3, line 27) for administration of hydrolyzed milk protein. The reference also suggests (col 3, line 47; col 5, line 56) administration of glutamine in addition to the hydrolyzed milk protein. Gray does not disclose that glutamine will promote recovery of an organ. Panigrahi discloses that glutamine will promote recovery of the intestines. Panigrahi does not disclose administration of hydrolyzed milk proteins.

In response, applicants have argued that Gray does not disclose that varying the degree of hydrolysis has the effect of altering the extent to which benefit accrues to one specific organ versus another. While applicants may be correct on this point, the claims are not drawn to a method of varying the degree of hydrolysis, or a Jepson claim in which the improvement is varying the degree of hydrolysis. The rejection is maintained.

✦

Claims 30, 32, 35 and 37-41 are rejected under 35 U.S.C. §103 as being unpatentable over Boza, Julio (*Journal of Pediatric Gastroenterology and Nutrition* **22**(2) 186-193, 1996).

Boza discloses that the weight and protein content of the jejunum mucosa is reduced following starvation, and that the hydrolase activity of the mucosa also is also reduced in starvation. Boza also discloses that these effects of starvation are reversed following administration of hydrolyzed milk proteins. Boza does not disclose that administration of hydrolyzed milk proteins will promote recovery of an organ.

In response, applicants have argued that Boza does not disclose that varying the degree of hydrolysis has the effect of altering the extent to which benefit accrues to one specific organ versus another. While applicants may be correct on this point, the claims are not drawn to a method of varying the degree of hydrolysis, or a Jepson claim in which the improvement is varying the degree of hydrolysis.

Applicants have also argued that Boza teaches only recovery of organs in subjects which have been subject to starvation. While this may be true, there is nothing in the instant claims to exclude this embodiment. The instant claims impose no limitations on the <u>cause</u> of the damage to the organs (which damage one is endeavoring to reverse).

Applicants have also argued that the degree of hydrolysis of the Boza hydrolyzates is not "pre determined". However, applicants have provided no evidence that this is the case, or even provided an argument as to what physical characteristics distinguish a hydrolyzate that has a "predetermined" degree of hydrolysis from a hydrolyzate in which the degree of hydrolysis is <u>not</u> "predetermined".

The rejection is maintained.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a). The practice of automatically extending the shortened statutory period an additional month upon filing of a timely first response to a final rejection has been discontinued by the Office. See 1021 TMOG 35.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED AND ANY EXTENSION FEE PURSUANT TO 37 CFR 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to David Lukton whose telephone number is 571-272-0952. The examiner can normally be reached Monday-Friday from 9:30 to 6:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell, can be reached at (571)272-0974. The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 571-272-1600.

DAVID LUKTON
PATENT EXAMINER
GROUP 1800

O I P E IAP2
MAR 0 7 2006
PATENT & TRADEMARK OFFICE

Notice of References Cited

Application/Control No.	Applicant(s)/Patent Under Reexamination	
09/508,635	BALLEVRE ET AL.	
Examiner	Art Unit	Page 1 of 1
David Lukton	1654	

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,048,543	04-2000	Schneider et al.	424/442
*	B	US-5,071,867	12-1991	Ichikawa et al.	514/408
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/508,635	05/18/2000	OLIVIER BALLEVRE	P00.0164	7617

29157 7590 01/30/2006

BELL, BOYD & LLOYD LLC
P. O. BOX 1135
CHICAGO, IL 60690-1135

EXAMINER	
LUKTON, DAVID	

ART UNIT	PAPER NUMBER
1654	

DATE MAILED: 01/30/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

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PATENT & TRADEMARK OFFICE

Advisory Action Before the Filing of an Appeal Brief	Application No.	Applicant(s)
	09/508,635	BALLEVRE ET AL.
	Examiner	**Art Unit**
	David Lukton	1654

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 11 January 2006 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:
   a) ☐ The period for reply expires ______months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.
   Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☒ The Notice of Appeal was filed on *11 January 2006*. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☒ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   (a)☒ They raise new issues that would require further consideration and/or search (see NOTE below);
   (b)☐ They raise the issue of new matter (see NOTE below);
   (c)☒ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   (d)☐ They present additional claims without canceling a corresponding number of finally rejected claims.
   NOTE: _____. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): a) ☒ will not be entered, or b) ☐ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.
   The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: *none*.
   Claim(s) objected to: *none*.
   Claim(s) rejected: *30,32,35 and 37-41*.
   Claim(s) withdrawn from consideration: *33 and 34*.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: See Continuation Sheet.
12. ☐ Note the attached Information Disclosure Statement(s). (PTO/SB/08 or PTO-1449) Paper No(s). _____
13. ☐ Other: _____.

D. Lukton

DAVID LUKTON
PATENT EXAMINER
GROUP 1800

Continuation of 11. does NOT place the application in condition for allowance because: Applicants have offered no new arguments, referring only to arguments previously made.

Antihypertensive Effect of Sour Milk and Peptides Isolated from It That are Inhibitors to Angiotensin I-Converting Enzyme

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ABSTRACT

This study reports the antihypertensive effect of orally administered doses of either Calpis sour milk or peptides (Val-Pro-Pro and Ile-Pro-Pro), which are inhibitors to angiotensin I-converting enzyme, isolated from the sour milk using strain SHR spontaneously hypertensive rats. Single oral administration of the sour milk (5 ml/kg of BW), corresponding inhibitory units of the peptides Val-Pro-Pro (.6 mg/kg of BW), or Ile-Pro-Pro (.3 mg/kg of BW) significantly decreased the systolic blood pressure from 6 to 8 h after administration. Blood pressure returned to the initial level at 24 h after administration. Antihypertensive activity of these two tripeptides was dose-dependent up to 5 mg/kg of BW. Conversely, the sour milk (25 ml/kg of BW) and mixed tripeptides (10 mg each of Val-Pro-Pro and Ile-Pro-Pro/kg of BW) did not change the systolic blood pressure of the normotensive strain WKY Wistar-Kyoto rats.
(**Key words**: angiotensin I-converting enzyme inhibitor, antihypertensive effect, sour milk)

Abbreviation key: ACE = angiotensin I-converting enzyme, SBP = systolic blood pressure.

INTRODUCTION

In the regulation of blood pressure, angiotensin I-converting enzyme (ACE) plays an important role in increasing blood pressure. In the renin angiotensin system, ACE acts on angiotensin I to hydrolyze His-Leu from its C-terminal and produces angiotensin II, which exhibits a strong vasoconstricting action (17). In addition, in the kinin kallikrein system, ACE inactivates bradykinin, which exhibits vasodilation action (6). Some inhibitors of this enzyme showed antihypertensive effects in vivo (12, 16). Several ACE inhibitory peptides were isolated and identified from food materials (3, 13, 18, 21). Yamamoto et al. (20) reported that the milk fermented by several strains of *Lactobacillus helveticus* has antihypertensive activity in spontaneously hypertensive rats (strain SHR). They (19) also reported the antihypertensive activity in SHR rats of casein, hydrolyzed by the cell-wall–associated proteinase from *L. helveticus* CP790, which was isolated from the Calpis sour milk (a Japanese fermented milk prepared by skim milk with a starter culture containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*). Yamamoto et al. (19) identified several peptides that had ACE inhibitory activity. However, no antihypertensive substance has been directly identified from a fermented milk. Nakamura et al. (15) recently isolated and identified two kinds of ACE inhibitory peptides, Val-Pro-Pro and Ile-Pro-Pro, from the Calpis sour milk. These two peptides represented most of the ACE inhibitory activity in the sour milk (15). The objective of the present study was to determine whether the sour milk and two ACE inhibitory peptides isolated from it show antihypertensive activities in SHR rats and also in normotensive strain WKY Wistar-Kyoto rats.

MATERIALS AND METHODS

Preparation of Calpis Sour Milk and Tripeptides

Calpis sour milk was prepared by fermenting 9% (wt/wt) reconstituted skim milk powder

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Accepted January 23, 1995.

(Yotsuba, Hokkaido, Japan) with the starter culture containing *L. helveticus* and *S. cerevisiae*, as previously described (15). Two kinds of tripeptides, Val-Pro-Pro and Ile-Pro-Pro, were chemically synthesized by the liquid-phase method.

Assay of ACE Inhibitory Activity

The ACE inhibitory activity was assayed by the method of Cushman and Cheung (5), modified as previously reported (15). The sour milk was centrifuged at 6000 × *g* for 10 min, and the supernatant fraction was used for the assay after the adjustment of pH to 8.3 by the addition of 10*N* NaOH. One unit of ACE inhibitory activity was determined as previously reported (15).

Rats

The spontaneously hypertensive, strain SHR male rats and male Wistar-Kyoto strain WKY rats, 20 wk of age, were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The rats received a laboratory diet (CE-2; Clea Japan, Tokyo, Japan) and tap water for ad libitum intake. All rats were housed in a room under controlled lighting from 0800 to 2000 h at 24 ± 1°C and relative humidity at 55 ± 5%.

Measurement of Blood Pressure

Rats were given the sour milk or the synthesized peptides dissolved in .2 m*M* PBS (pH 7.2) containing .5% sodium caseinate by gastric intubation. Control rats were given the unfermented milk or the PBS-caseinate alone. After rats were kept at 45°C for 5 min, systolic blood pressure (SBP) was measured by tail-cuff method with a programmed electrosphygmomanometer (PE-300; NARCO Bio-Systems, Austin, TX).

Statistical Analysis

Change of SBP was expressed as the difference of SBP before and after administration. Data are expressed as means and standard errors. Student's *t* test was used for statistical analysis.

RESULTS

Antihypertensive Effect

When the SBP was measured at 22 to 26 wk of age, the BW of SHR and WKY rats were 330 to 400 g and 350 to 390 g, respectively. Figure 1 shows the change of the SBP after administration of the sour milk or unfermented milk in SHR rats. The mean SBP was 213.7 ± 3.4 mm Hg before administration. At 4, 6, and 8 h after administration of 5 ml of sour milk/kg of BW, the SBP significantly decreased by 20.0 ± 5.2 ($P < .05$), 21.8 ± 4.2 ($P < .05$), and 17.7 ± 3.5 mm Hg ($P < .05$), respectively. Then the SBP returned to the initial SBP at 24 h after administration. The SBP of rats given 5 ml of unfermented milk/kg of BW did not change significantly.

Figure 2 shows the change of SBP after administration of .6 mg of Val-Pro-Pro/kg of BW or .3 mg of Ile-Pro-Pro/kg of BW, which equals the administration of 5 ml of sour milk/kg of BW in the extent of ACE inhibitory activity (750 U/kg of BW). The mean SBP was 230.1 ± 3.3 mm Hg before administration. At 2, 4, 6, and 8 h after administration of Val-Pro-Pro, the SBP significantly decreased by 24.6 ± 3.5 ($P < .001$), 32.1 ± 5.3 ($P < .01$), 28.8 ± 3.6 ($P < .01$), and 26.7 ± 5.3 mm Hg ($P < .01$), respectively. At 2, 4, 6, and 8 h after administration of Ile-Pro-Pro, the SBP significantly decreased by 16.2 ± 5.8 ($P < .05$), 21.7 ± 4.1 (P

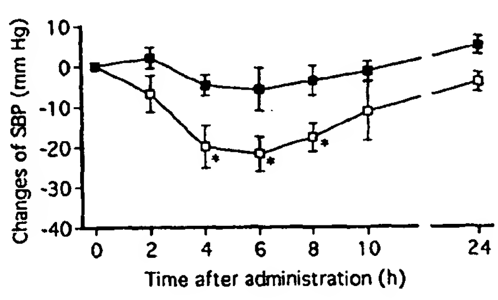

Figure 1. Effect of a single oral administration (time 0) of the sour milk on strain SHR spontaneously hypertensive rats. Each point indicates the mean of the systolic blood pressure (SBP) of five SHR rats, and the vertical bars represent the standard errors. Treatments were control (■; 5 ml of unfermented milk/kg of BW) and 5 ml of sour milk/kg of BW (□). Significant difference from control: *$P < .05$.

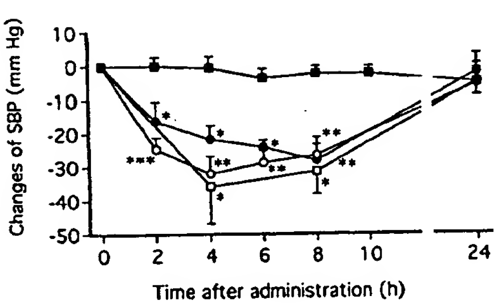

Figure 2. Effect of single oral administration (time 0) of Val-Pro-Pro or Ile-Pro-Pro on strain SHR spontaneously hypertensive rats. Each point indicates the mean of the systolic blood pressure (SBP) of four rats, and the vertical bars represent the standard errors. Treatments were control (■; PBS-caseinate alone), .6 mg of Val-Pro-Pro/kg of BW (O), .3 mg of Ile-Pro-Pro/kg of BW (●), and 5 ml of sour milk/kg of BW (□). Significant difference from control: *P < .05, **P < .01, and ***P < .001.

< .05), 24.1 ± 2.2 (P < .05), and 28.3 ± 4.8 mm Hg (P < .05), respectively. In both of the groups treated with the peptides, the SBP returned to the initial level at 24 h after administration. The SBP of rats given PBS-caseinate alone did not change significantly.

Dose Effect of Val-Pro-Pro and Ile-Pro-Pro

Figure 3 shows the dose effects of Val-Pro-Pro and Ile-Pro-Pro on the SBP of SHR rats. The mean SBP was 215.4 ± 1.3 mm Hg before administration. The changes of SBP were measured 6 h after the single oral administration of Val-Pro-Pro or Ile-Pro-Pro. After administration of .4, 1.6, and 5 mg of Val-Pro-Pro/kg of BW, SBP was significantly decreased by 11.2 ± 3.0 (P < .05), 20.0 ± 5.3 (P < .05), and 20.1 ± 1.6 mm Hg (P < .001), respectively. Similarly, when 1 and 5 mg of Ile-Pro-Pro/kg of BW were administered, SBP was significantly decreased by 15.1 ± 3.6 (P < .05) and 18.3 ± 3.7 mm Hg (P < .01). Both peptides showed dose-dependent activity up to the dosage of 5 mg/kg of BW, the highest dose.

Effect in Normotensive Rats

Figure 4 shows the effects of oral administration of sour milk or tripeptides on the SBP in normotensive WKY rats. The mean SBP in WKY rats was 134.7 ± 1.3 mm Hg (n = 15). The SBP of rats given sour milk (25 ml/kg of BW), mixed tripeptides (10 mg each of Val-Pro-Pro and Ile-Pro-Pro/kg of BW), and the control sample (PBS-caseinate) did not change significantly.

DISCUSSION

We reported in our preceding paper (15) that most of the ACE inhibitory activity of the Calpis sour milk was attributed to the two kinds of tripeptides, Val-Pro-Pro and Ile-Pro-Pro. By administration of corresponding ACE inhibitory activity of the sour milk or tripeptides to SHR rats, the extent of decrease in the SBP was similar several hours after administration (Figure 2). The antihypertensive activities of these peptides were further confirmed by the dose-effect experiment (Figure 3). These results indicated that both Val-Pro-Pro and Ile-Pro-Pro play an essential role in the antihypertensive activity of the Calpis sour milk.

Some differences existed in the mean SBP in SHR rats and in the extent of decrease in the SBP after administration of 5 ml of sour milk/kg of BW among the different series of experiments (Figures 1, 2, and 3). A similar tendency was observed for Val-Pro-Pro and Ile-Pro-Pro (Figures 2 and 3). Each series of experiments occurred at different times using rats from different breeding lots. These differences might have affected the SBP and sensitivity of rats to the sour milk and the tripeptides. However, the extent of decrease in SBP after the administration of corresponding ACE inhibitory activity of the sour milk and the tripeptides was similar for the same series of experiments (Figures 2 and 3).

The amount of the Val-Pro-Pro and Ile-Pro-Pro that was required to show antihypertensive effects is relatively small compared with other ACE inhibitory peptides (8, 11). Small peptides, such as di- or tripeptides, are more easily absorbed in the intestine than are amino acids or larger oligopeptides (1, 4, 7). In addition, peptides containing Pro are generally resistant to the degradation by digestive enzymes (2, 9). Although several peptidases that are specific for Pro are reported, the tripeptides that have Pro-Pro sequence at the C-terminal are resistant to the degradation by peptidase (10, 14, 22, 23). Therefore, these tripeptides may be resistant to digestion, efficiently absorbed in

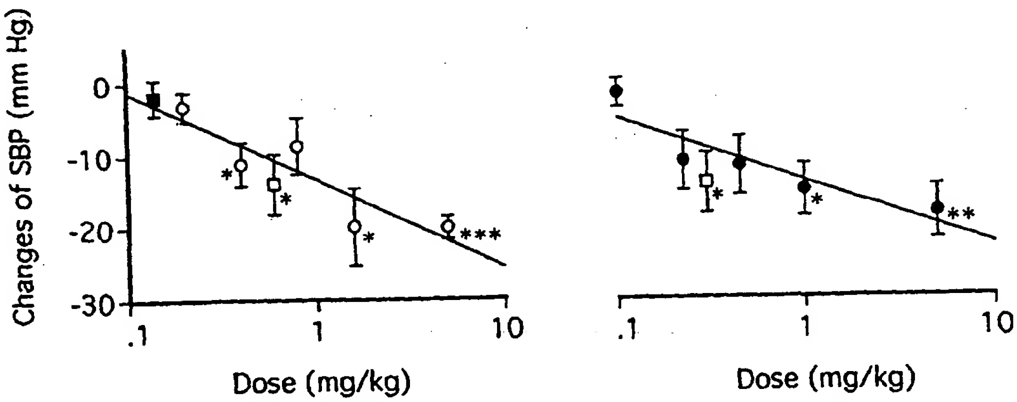

Figure 3. Dose effect of Val-Pro-Pro (left) or Ile-Pro-Pro (right) on strain SHR spontaneously hypertensive rats. The systolic blood pressure (SBP) of rats was measured 6 h after single oral administration. Each point indicates the mean of the SBP of 4 to 6 rats, and the vertical bars represent the standard errors. Treatments were control (■; PBS-caseinate); .2, .4, .8, 1.6, and 5 mg of Val-Pro-Pro/kg of BW (○); .1, .2, .4, 1, and 5 mg of Ile-Pro-Pro/kg of BW (●); and 5 ml of sour milk/kg of BW (□). The data for sour milk are indicated at the dose of corresponding angiotensin-I converting enzyme inhibitory units of the peptides, Val-Pro-Pro (.6 mg/kg of BW) and Ile-Pro-Pro (.3 mg/kg of BW). The line for values of Val-Pro-Pro illustrates the equation: $y = -13.359$ (SE = 3.734) $- 12.218$ (SE = 3.453) (x); $r^2 = .807$. The line for values of Ile-Pro-Pro illustrates the equation: $y = -13.754$ (SE = 2.976) $- 8.974$ (SE = 2.287) (x); $r^2 = .837$. Significant difference from control: *$P < .05$, **$P < .01$, and ***$P < .001$.

the intestine, and hence, exhibit the antihypertensive effect. In contrast, administration of the sour milk (25 ml/kg of BW) or mixed tripeptides, Val-Pro-Pro (10 mg/kg of BW) and Ile-Pro-Pro (10 mg/kg of BW), did not change SBP of normotensive WKY rats (Figure 4), even though the amounts of the sour milk and the tripeptides administered were far higher than the dose that is effective in SHR rats. These results suggested that the antihypertensive activity of the sour milk and the tripeptides, Val-Pro-Pro and Ile-Pro-Pro, was specific to the hypertensive state.

However, further studies are necessary to clarify the detailed mechanism of the effects in vivo and in effects in humans.

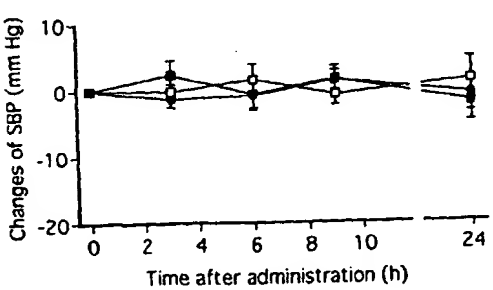

Figure 4. Effect of single oral administration (time 0) of the sour milk or the tripeptides on strain WKY Wistar-Kyoto rats. Each point indicates the mean of the systolic blood pressure (SBP) of five rats, and the vertical bars represent the standard errors. Treatments were control (■; PBS-caseinate), 25 ml of sour milk/kg of BW (□), and a mixture of 10 mg of each of Val-Pro-Pro and Ile-Pro-Pro/kg of BW (●).

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Antihypertensive Peptides Are Present in Aorta after Oral Administration of Sour Milk Containing These Peptides to Spontaneously Hypertensive Rats[1,2]

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ABSTRACT Absorption of inhibitory peptides to angiotensin I-converting enzyme, L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline, was studied in spontaneously hypertensive rats and normotensive Wistar-Kyoto rats after an oral administration of Calpis™ sour milk, which contains these peptides. Six hours after the administration of Calpis™ sour milk or saline to spontaneously hypertensive rats or normotensive Wistar-Kyoto rats, the blood pressure was measured and rats were killed. Abdominal aorta, lung, kidney, heart and brain were excised, homogenized and solubilized by detergent treatments. Angiotensin I-converting enzyme activity in the solubilized fractions from the abdominal aorta was significantly lower in spontaneously hypertensive rats given the sour milk than in the rats given saline. L-Valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline were detected by HPLC in the heat-treated solubilized fraction from the abdominal aorta of spontaneously hypertensive rats given the sour milk. In contrast, the tripeptides were not detected in rats given saline, or in normotensive Wistar-Kyoto rats given the sour milk or saline. These data suggest that L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline in the Calpis™ sour milk are absorbed directly without being decomposed by digestive enzymes, reach the abdominal aorta, inhibit the angiotensin I—converting enzyme, and show antihypertensive effects in spontaneously hypertensive rats. J. Nutr. 126: 3063–3068, 1996.

INDEXING KEY WORDS:

- *antihypertensive peptide* • *fermented milk*
- *angiotensin I–converting enzyme*
- *spontaneously hypertensive rats*
- *intestinal absorption*

Angiotensin I-converting enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1)[4] plays an important role in regulating blood pressure. It catalyzes the formation of potent vasoconstrictor angiotensin II by cleaving dipeptide from the C-terminal of angiotensin I (Skeggs et al. 1956), and also inactivates the vasodilator, bradykinin (Erdos 1975). Generation of angiotensin II in tissues such as vascular wall and kidney has been demonstrated (Ganten et al. 1976, Giasson et al. 1981, Miyazaki et al. 1984, Re et al. 1982). It has been suggested that inhibition of ACE in the vascular wall, kidney, brain and lung is important for the expression of antihypertensive activity of Captopril, an ACE inhibitory medicine (Unger et al. 1981 and 1985, Velletri and Bean 1982).

Nakamura et al. (1995a) isolated and identified two kinds of ACE inhibitory tripeptides, L-valyl-L-prolyl-L-proline (Val-Pro-Pro) and L-isoleucyl-L-prolyl-L-proline (Ile-Pro-Pro), from Calpis™ sour milk, which is prepared by fermenting milk with a starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae.* These two tripeptides are formed in the sour milk during fermentation and are responsible for most of the ACE inhibitory activity of the sour milk (Nakamura et

[1] Presented in abstract form at the plenary meeting of the Japanese Society of Nutrition and Food Science, 1995, Gifu, Japan (Masuda, O., Nakamura, Y. & Takano, T. (1995) Detection of ACE inhibitory peptides from organs after oral administration of sour milk containing these peptides (in Japanese)).

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[4] Abbreviations used: ACE, angiotensin I–converting enzyme; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; Ile-Pro-Pro, L-isoleucyl-L-prolyl-L-proline; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TFA, trifluoroacetic acid; Val-Pro-Pro, L-valyl-L-prolyl-L-proline.

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al. 1995a). The sour milk and tripeptides show antihypertensive effects after a single oral administration to spontaneously hypertensive rats (SHR) (Nakamura et al. 1995b).

Although many ACE inhibitory peptides produced by enzymatic digestion of food materials were reported (Maruyama et al. 1985 and 1989, Matsui et al. 1993, Yokokawa et al. 1992), little is known about their action in vivo after oral administration. Recently, we found that the systolic blood pressure (SBP) and ACE activity in aorta were significantly lower after long-term feeding of the sour milk in SHR rats than in rats fed a control diet (Nakamura et al. 1996). However, the presence of these tripeptides in the aorta was not directly demonstrated. In the present study, we demonstrated the decrease of ACE activity and also the presence of ACE inhibitory tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the aorta after a single oral administration of Calpis™ sour milk to SHR rats.

MATERIALS AND METHODS

Preparation of sour milk. Calpis™ sour milk was prepared by fermenting 9 g/100 g reconstituted skim milk powder (Yotsuba, Hokkaido, Japan) with a starter culture containing *L. helveticus* and *S. cerevisiae* as previously described (Nakamura et al. 1995a). Two kinds of ACE inhibitory tripeptides, Val-Pro-Pro and Ile-Pro-Pro, were prepared as follows. Unsoluble proteins were removed from 2 mL of the sour milk by centrifugation. The whey fraction was applied to a column of Amberlite XAD-7 (0.9 × 3.0 cm, ORGANO, Tokyo, Japan) connected to a Sep-pak C18 column (Waters, Tokyo, Japan) and washed with 5 mL of distilled water. The adsorbed substances on the Sep-pak C18 column were eluted with 5 mL of 800 g/L MeOH, concentrated with a centrifugal concentrator, and lyophilized. The dried fraction was dissolved with 0.5 g/L trifluoroacetic acid (TFA) solution containing 0.3 mol/L NaCl, and subjected to HPLC analysis using gel filtration column (Asahipak GS320; Asahi Chemical, Tokyo, Japan) and the same solution for elution at a flow rate of 0.5 mL/min. The tripeptides were monitored at 215 nm and quantified using chemically synthesized peptides as a standard.

Experimental animals. Animals used in this study were maintained in accordance with the guidelines of the Institutional Animal Care and Utilization Committee in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Eight male SHR rats and eight male strain WKY Wistar-Kyoto rats, 18 wk of age, were obtained from Charles River Japan (Kanagawa, Japan). All rats had free access to a nonpurified diet (CE-2; Clea Japan, Tokyo, Japan) and also to tap water. The CE-2 diet is a crude diet containing moisture (89.3 g/kg), crude protein (254 g/kg), crude fat (44.3 g/kg), crude fiber (41.3 g/kg), and crude ash (69.3 g/kg). The energy value of this diet is 1.7 kJ/g. All rats were housed in a room under controlled lighting 0800–2000 h at 24 ± 1°C and relative humidity at 55 ± 5%.

Measurement of blood pressure. Each of four SHR and WKY rats were given 10 mL of the sour milk or saline per kilogram of body weight by gastric intubation. The change in SBP was measured 6 h after administration of the sour milk or saline by the tail cuff method using a programmed electrosphygmomanometer (model BP-98A; Softron, Tokyo, Japan).

Preparation of tissue enzyme extract. Rats were killed immediately after the measurement of SBP by exsanguination from the inferior vena cava while under sodium pentobarbital anesthesia. The enzyme extracts of abdominal aorta, lung, kidney, heart and brain were prepared by the method of Das and Soffers (1975) with some modifications. All organs were chopped into small pieces and homogenized in 50 mmol/L Tris-HCl (pH 7.9) containing 0.3 mol/L NaCl (2 mL per g organ in aorta, and 4 mL per g organ in lung, kidney, heart and liver) using an ultra disperser (model LK-21; Yamato, Tokyo, Japan). A sequence of 30 s of dispersing followed by 1 min of resting on ice was followed. The suspension was filtered through nylon mesh (No. 20; Abe Chemical, Tokyo, Japan). The filtrates were centrifuged at 44,000 × *g* for 90 min and the supernatants were discarded. Pellets were suspended in the same buffer (2 mL per g of original tissue in aorta, 4 mL per g of original tissue in lung, kidney, heart and brain) and centrifuged at 44,000 × *g* for 90 min; the supernatants were discarded. The pellets were resuspended in the above buffer containing 0.5% Triton X-100 (2 mL per g of original tissue in aorta, 4 mL per g of original tissue in lung, kidney, heart and brain). After 1 h, the suspensions were centrifuged at 1000 × *g* for 10 min and the supernatants were designated the detergent solubilized ACE fraction.

Enzyme assays. ACE activity was determined using hippuryl-L-histidyl-L-leucine (Hip-His-Leu; Sigma, St. Louis, MO) as a substrate according to the method of Cushman and Cheung (1971). Hip-His-Leu was dissolved in 0.1 mol/L sodium borate buffer (pH 8.3) containing 0.3 mol/L NaCl. After preincubation of 150 μL of 5 mmol/L Hip-His-Leu solution for 3 min at 37°C, the reaction was initiated by addition of 100 μL of the enzyme extract or serum, and the mixture was incubated for 30 min at 37°C. The reaction was stopped by addition of 250 μL of 1 mol/L HCl. The hipurric acid liberated by ACE was extracted with 1.7 mL ethyl acetate, dissolved by addition of 1 mL of distilled water after removal of ethyl acetate by heating for 30 min at 120°C, and measured spectrophotometrically at 228 nm. One unit (U) of activity was defined as the amount of enzyme which released 1.0 mmol of hippuric acid per min under these conditions. The specific activity was expressed as units/mg protein.

Detection of ACE inhibitors in tissues. Each enzyme extract was analyzed for Val-Pro-Pro and Ile-Pro-Pro by a method essentially similar to that used in the analysis of the sour milk described above. Each detergent-solubilized enzyme extract was heated for 5 min in boiling water. After cooling, the extract was combined with 50 g/L trichloroacetic acid and centrifuged at 10,000 × g for 10 min. The supernatant (1 mL for aorta, 5 mL for lung, kidney, heart and brain) was applied to a column of Amberlite XAD-7 connected to a Sep-pak C18 column and washed with 5 mL of distilled water. The adsorbed substances on the Sep-pak C18 column were eluted with 5 mL of 80% MeOH. The eluted fraction was concentrated with a centrifugal concentrator and lyophilized. The dried fraction was dissolved with 1 mL of 50 mmol/L sodium acetate buffer (pH 4.0), applied on the Sep-pak CM column (Waters) and washed with 5 mL of the same buffer. The eluted fraction was collected and lyophilized. The dried fraction was dissolved with 200 μL of 50 mmol/L ammonium acetate buffer (pH 6.89), applied to HPLC using a gel filtration column (Asahipak GS320HQ; Asahi Chemical) and eluted with the same buffer at a flow rate of 0.6 mL/min. Elution was monitored at 215 nm. After a retention time of 16–19 min, a fraction which is supposed to contain Val-Pro-Pro and Ile-Pro-Pro (Nakamura et al. 1995a), was collected and lyophilized. The dried fraction was dissolved with 1 mL of 1 g/L TFA solution, applied to HPLC using a reverse-phase column (Bondasphere C18; Waters) and eluted with linear gradient of acetonitrile (0–20% for 30 min) containing 0.7 g/L TFA at a flow rate of 1.0 mL/min.

Protein determination. Protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin (Type V; Sigma) as a standard.

Amino acid analysis. Amino acid composition was analyzed using an HPLC system (JASCO PU-980; JASCO, Tokyo, Japan) after hydrolysis in 6 mol/L HCl containing 0.1% phenol for 24 h at 110°C. Amino acid sequence was analyzed by a protein sequencer (PPSQ-10; Shimadzu, Kyoto, Japan).

Statistical analysis. Change in SBP was expressed as the difference in SBP before and after administration. Student's *t* test was used for statistical analysis of the change in SBP. The difference for ACE activity in rats was tested by use of Tukey's test (Steel and Torrie 1982) after two-way ANOVA using the QCAS computer program (The Institute of JUSE, Tokyo, Japan). Data are expressed as means and SEM; $P < 0.05$ was considered significant.

RESULTS

ACE activity in various organs. The concentrations of moisture, protein, ash and carbohydrate in the sour milk were 915, 31, 7 and 47 mg/g sour milk respectively; the energy value was 1.30 kJ/g sour milk. Concentrations of Val-Pro-Pro and Ile-Pro-Pro in the sour milk were 21.7 and 14.1 mg/L, respectively. When the SHR and WKY rats were administered the sour milk at 23 wk of age, their body weights were 350–370 and 340–360 g, respectively. In SHR rats, 6 h after the administration of the sour milk, the SBP was significantly decreased by 26.4 ± 3.1 mm Hg ($P < 0.01$). In contrast, the SBP in WKY rats did not change significantly after the administration of the sour milk (−5.3 ± 3.9 mm Hg). In both SHR and WKY rats, the SBP did not change significantly after the administration of saline: +7.6 ± 5.4 and −4.2 ± 5.7 mm Hg, respectively. At 6 h, all rats were killed to measure ACE activity in various organs. ACE activity in abdominal aorta and lung extracts from SHR rats given saline as significantly higher ($P < 0.01$ and 0.05, respectively) than activity from WKY rats given saline (Fig. 1). ACE activity in aorta extract from SHR rats given the sour milk was significantly lower ($P < 0.05$) than that from SHR rats given

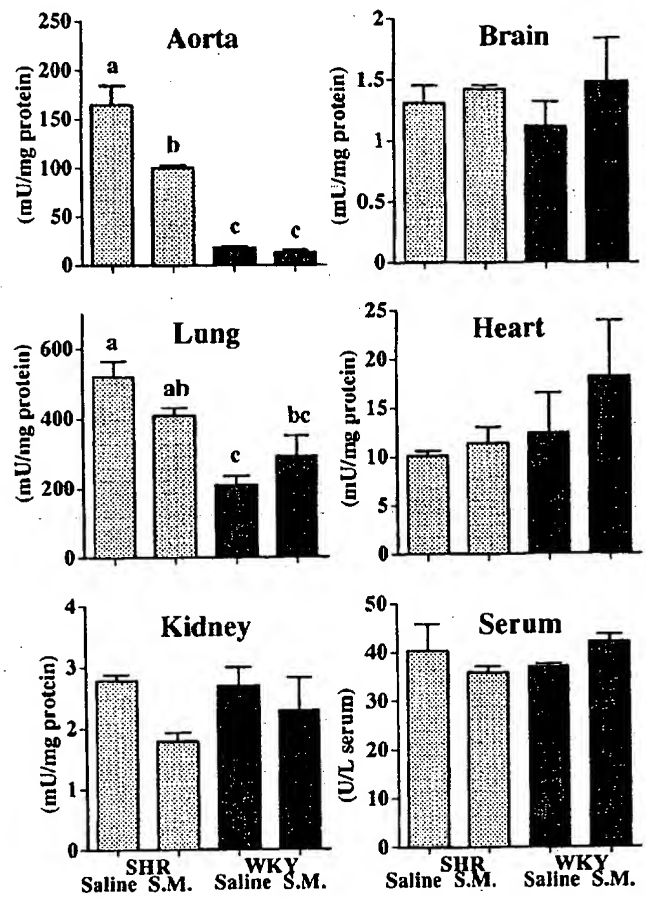

FIGURE 1 The activity of angiotensin I-converting enzyme (ACE) in serum and organs from SHR spontaneously hypertensive rats and strain WKY Wistar-Kyoto rats administered saline or Calpis™ sour milk (S.M.). Six hours after administration, ACE activity was measured. Values are expressed as means ± SEM of 4 or 5 rats. Values with different letters are significantly different, P < 0.05.

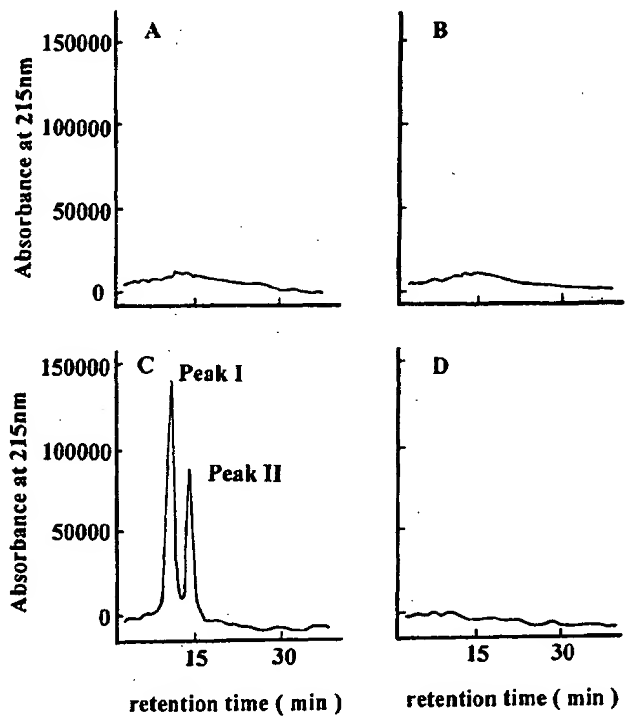

FIGURE 2 The chromatogram at the final purification step of L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline from serum (*panels A* and *B*) and aorta (*panels C* and *D*) of strain SHR spontaneously hypertensive rats after administration of saline (*panels B* and *D*) and sour milk (*panels A* and *C*).

saline. The ACE activity in lung extract from SHR rats given the sour milk was slightly ($P = 0.08$), lower than that from SHR rats given saline (Fig. 1). After administration of the sour milk to WKY rats, no significant difference from controls was observed in ACE activity in the extract from any organ (Fig. 1).

Detection of tripeptides in tissue. Preliminary experiments showed that the recovery of Val-Pro-Pro and Ile-Pro-Pro at each step of purification was about 95% or greater (data not shown). Two peaks (peaks 1 and 2) were detected in the HPLC chromatogram at the final purification step in the extract of abdominal aorta from SHR rats given the sour milk (**Fig. 2**). No obvious peaks were observed in extracts of other organs, including serum (Fig. 2) from the same rats. No peaks were observed in any organs from SHR rats given saline or from WKY rats given the sour milk or saline.

Amino acid composition and the sequence of peaks 1 and 2 from four SHR rats given the sour milk are shown in **Table 1.** Peak 1 consisted of two kind of amino acids, valine and proline, and their ratio was about 1:2. The amino acid sequence of peak 1 was Val-Pro-Pro. Peak 2 also consisted of two kind of amino acids, isoleucine and proline, and their ratio was also about 1:2. The amino acid sequence of peak 2 was Ile-Pro-Pro. The amount of Val-Pro-Pro and Ile-Pro-Pro detected in the enzyme extract of abdominal aorta was 4.3 ± 1.5 and 3.5 ± 1.6 μg/rat, respectively.

DISCUSSION

Six hours after the administration of Calpis™ sour milk, the SBP of SHR rats was significantly decreased as previously reported (Nakamura et al. 1995b). Specific activity of ACE in aorta extract was significantly lower in rats given the sour milk than in those given saline (Fig. 1). Recently, we found that the SBP increase was inhibited and ACE activity in abdominal aorta was significantly lower in SHR rats fed Calpis™ sour milk for 18 wk compared with rats fed a control diet (Nakamura et al. 1996). Present results indicate that a similar decrease in SBP and ACE activity in aorta takes place after a single oral administration of the sour milk. When ACE activity in various organs of SHR and WKY rats was compared, the activity in abdominal aorta and lung was significantly higher in SHR rats (Fig. 1) as has been previously reported (Okunishi et al. 1991). The higher ACE activity in abdominal aorta, lung and kidney is thought to be responsible for the hypertension of SHR rats (Chevillard et al. 1988, Cohen et al. 1983, Inada et al. 1986, Okunishi et al. 1991, Unger et al. 1984, Velletri and Bean 1982). Present results and these previous reports suggest that inhibition of ACE in aorta may play an important role in the antihypertensive effect of the sour milk.

The ACE inhibitory tripeptides were detected in the abdominal aorta after oral administration of the sour milk to SHR rats (Fig. 2, Table 1). These tripeptides are not found in the aorta of SHR rats given saline. Similar results were obtained when unfermented acidified milk, which was prepared by adding 22 g/L of D,L-lactic acid to 9 g/100 g reconstituted skim milk, was administered (data not shown). This suggests that the tripeptides are not formed from milk protein in the rat bodies. Although several peptidases that are specific for proline are known, the tripeptides that have the L-prolyl-L-proline sequence at the C-terminus are resistant to degradation by peptidases (Kim et al. 1972, Mock et al. 1990, Yoshimoto et al. 1978). Small peptides are more easily absorbed in the intestine than amino acids or larger oligopeptides (Adibi 1977, Hara et al. 1984, Matthews and Adibi 1976, Silk et al. 1980). It is likely that the tripeptides in the sour milk were not decomposed by digestive enzymes and were therefore absorbed and delivered to the aorta in their intact form.

Average yields per rat of the tripeptides were 4.3 g and 3.5 μg for Val-Pro-Pro and Ile-Pro-Pro, respectively. This corresponds to the recovery of 4.0 and 5.0% of tripeptides ingested, respectively. Although there have been reports that radioisotope-labeled ACE inhibitory substances were detected in tissue and serum (Jarrott et al. 1982, Kripalani et al. 1980), this is the first obser-

TABLE 1

Amino acid composition and sequence of peaks 1 and 2 isolated from abdominal aorta of spontaneously hypertensive rats (SHR) after a single oral administration of Calpis™ sour milk[1]

Individual rat	Peak[2]	Composition[3]	Sequence[4]	Content[5] (µg/rat)
1	1	Val: Pro = 1.00: 2.07	Val-Pro-Pro	5.6
	2	Ile-Pro = 1.00: 2.11	Ile-Pro-Pro	4.7
2	1	Val: Pro = 1.00: 2.09	Val-Pro-Pro	3.2
	2	Ile: Pro = 1.00: 2.14	Ile-Pro-Pro	2.4
3	1	Val: Pro = 1.00: 2.05	Val-Pro-Pro	5.4
	2	Ile: Pro = 1.00: 2.14	Ile-Pro-Pro	5.1
4	1	Val: Pro = 1.00: 2.12	Val-Pro-Pro	3.1
	2	Ile: Pro = 1.00: 2.05	Ile-Pro-Pro	2.2

1 Four SHR rats were administered Calpis™ sour milk. The aorta of each rat was excised 6 h after administration and analyzed for L-valyl-L-prolyl-L-proline and L-isoleucyl-L-prolyl-L-proline as described in Materials and Methods.
2 Peaks 1 and 2 were obtained by µ Bondasphere C18 column chromatography.
3 Amino acid composition was analyzed using an HPLC system (JUSCO PU-980).
4 Amino acid sequence was analyzed using a protein sequencer (Shimadzu PPSQ-10).

vation that the biologically active peptides derived from food materials were detected in their target organ intact after oral administration.

The tripeptides were detected in aorta from SHR but not WKY rats given the sour milk. ACE activity in aorta from SHR rats was significantly higher than in aorta from WKY rats (Fig. 1). Although neither the ACE expression in aorta nor affinity of the tripeptides for ACE was measured directly, a detectable level of tripeptides might have attached to the ACE molecule in the aorta of SHR rats but not in a sufficient amount to be detectable in the WKY rats. When we make such an assumption, further questions are raised. For example, why were the tripeptides not detected in lung, an organ in which ACE activity was higher than that in aorta? More studies are required to understand fully the detailed metabolism of the tripeptides in vivo.

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(54) Title: CASEIN FRAGMENTS HAVING GROWTH PROMOTING ACTIVITY

(57) Abstract

Amino acid sequences substantially identical to the C-terminal end of an α-S2 casein precursor are shown to act as growth promoters. Disclosed are sequences from Bovine α-S2 casein including the 9 C-terminal amino acids: LysValIleProTyrValArgTyrLeu. Also disclosed are foodstuffs and medicaments comprising the peptides of the invention and a method of producing same.

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DESCRIPTION

CASEIN FRAGMENTS HAVING GROWTH PROMOTING ACTIVITY

The present invention relates to growth promoters.

It has long been known that milk contains growth promoting activity for cells that is additional to its nutritional content. Thus, Epidermal Growth Factor (EGF) has been identified in human (Shing and Klagsbrun, 1984, Petrides, 1985), rat (Raaberg et al, 1990), swine (Tan et al 1990) and goat (Brown and Blakeley, 1983) milk.

Indeed the EGF present in rat milk has been shown to be significant for the normal development of pups (Oka et al 1983). EGF has not, however, been found in bovine milk (Read 1985). Instead insulin-like growth factor (IGF) I and II (Francis et al, 1986) and bovine colostrum growth factor (BCGF), which is structurally related to Platelet-derived Growth Factor (PDGF) (Shing and Klagsbrun, 1984, Brown and Blakeley, 1984), have been identified.

The applicant has surprisingly discovered that bovine milk contains growth promoting activity for rat mammary fibroblast cell line (Rama 27), which is not significantly stimulated by IGF or PDGF.

Furthermore, they have identified peptide sequences which elicit this growth promoting activity.

The invention relates to a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of the α-S2 casein precursor.

According to a first aspect of the present invention there is provided the use of a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of an α-S2 casein precursor, for the manufacture of a medicament or foodstuff for promoting growth.

Whilst whole casein protein shows no growth activity, the applicant has identified a number of peptides, derived from the C-terminal end of Bovine α-S2 casein, which elicit growth promoting activity.

Indeed, the applicant has shown this growth promoting activity to be present in at least peptides of 9 to 31 amino acids in length which have been derived from the C-terminal end of Bovine α-S2 casein. It is reasonable to hypothesise that the natural sequence responsible for the growth promoting activity is the sequence comprising the last 9 amino acids of the C-terminal end or an even shorter sequence from within the nine amino acid sequence, possibly an 8 or 7 amino acid sequence. Indeed, it may be as short as a 3 amino acid sequence.

The bovine α-S2 casein precursor is characterised

in that it has an amino acid sequence:

[CAS2_BOVIN] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE

MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEKN MAINPSKENL CSTFCKEVVR
NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQKALNEI NQFYQKFPQY LQYLYQGPIV
LNPWDQVKRN AVPITPTLNR EQLSTSEENS KKTVDMESTE VFTKKTKLTE EEKNRLNFLK
KISQRYQKFA LPQYLKTVYQ HQKAMKPWIQ PKTKVIPYVR YL

In three letter codes this translates to:

[CAS2 BOVIN] ALPHA-S2 CASE IN PRECURSOR.
SEQUENCE

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLeuAsnThrMetGlu

HisValSerSerSerGluGluSerIleIle
SerGlnGluThrTyrLysGlnGluLysAsn

MetAlaIleAsnProSerLysGluAsnLeu
CysSerThrPheCysLysGluValValArg

AsnAlaAsnGluGluGluTyrSerIleGly
SerSerSerGluGluSerAlaGluValAla

ThrGluGluValLysIleThrValAspAsp
LysHisTyrGlnLysAlaLeuAsnGluIle

AsnGlnPheTyrGlnLysPheProGlnTyr
LeuGlnTyrLeuTyrGlnGlyProIleVal

LeuAsnProTrpAspGlnValLysArgAsn
AlaValProIleThrProThrLeuAsnArg

GluGlnLeuSerThrSerGluGluAsnSer
LysLysThrValAspMetGluSerThrGlu

ValPheThrLysLysThrLysLeuThrGlu
GluGluLysAsnArgLeuAsnPheLeuLys

LysIleSerGlnArgTyrGlnLysPheAla
LeuProGlnTyrLeuLysThrValTyrGln

HisGlnLysAlaMetLysProTrpIleGln
ProLysThrLysValIleProTyrValArg

TyrLeu

The applicant has found that short peptide sequences incorporating the C-terminal sequence -LysValIleProTyrValArgTyrLeu show growth promoting activity.

According to a second aspect of the present invention there is provided a growth factor comprising the amino acid sequence -LysValIleProTyrValArgTyrLeu

Furthermore, comparison of, for example, the last 20 amino acids of the C-terminal sequence for bovine α-S2 casein with those for goat, and sheep shows a high degree of homology as does to a lesser extent the C-terminal amino acid sequence of rabbit and pig α-S2 casein

The sequences for these are set out below.

```
[CAS2_CAPHI]    ALPHA-S2 CASEIN PRECURSOR (ALPHA-S2-CN).
SEQUENCE
   MKFFIFTCLL AVALAKHKME EVSSSEEPIN IFQEIYKQEK NMAIHPRKEK LCTTSCEEVV
   RNANEEEYSI RSSSEESAEV APEEIKITVD DKHYQKALNE INQFYQKFPQ YLQYPYQGPI
   VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
   KKISQYYQKF AWPQYLKTVD QHQKAMKPWT QPKTNAIPYV RYL
```

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>pir|S33881|S33881 alphas2-casein E - goat

MKFFIFTCLL AVALAKHKME HVSSSEEPIN IFQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNANEEEYSI RSSSEESAKV APEEIKITVD DKHYQKALNE INQFYQKFPQ YLQYPYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
KKISQYYQKF AWPQYLKTVD QHQKAMKPWT QPKTNAIPYV RYL 223
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>gp|S74171|S74171_1 alpha s2-casein C [Capra hircus]

MKFFIFTCLL AVALAKHKME HVSSSEEPIN IFQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNANEEEYSI RSSSEESAEV APEEIKITVD DKHYQKALNE INQFYQKFPQ YLQYPYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
KIISQYYQKF AWPQYLKTVD QHQKAMKPWT QPKTNAIPYV RYL 223
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>pir|S39776|S39776 alpha-S2-casein form b precursor - rabbit
>gp|X76909|OCPAS2BCS_1 pre-alpha S2b casein (AA -15 to 167)
[Oryctolagus cuniculus]

MKFFIFTCLL AVALAKPKIE QSSSEETIAV SQEVSPNLEN ICSTACEEPI KNINEVEYVE
VPTEIKDQEF YQKVNLLQYL QALYQYPTVM DPWTRAETKA IPFIRTMQYK QEKDATKHTS
QKTELTEEEK AFLKYLDEMK QYYQKFVFPQ YLKNAHHFQK TMNPWNHVKT IIYQSVPTL 179

[CAS2_SHEEP] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE
MKFFIFTCLL AVALAKHKME HVSSSEEPIN ISQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNADEEEYSI RSSSEESAEV APEEVKITVD DKHYQKALNE INQFYQKFPQ YLQYLYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
KKISQYYQKF AWPQYLKTVD QHQKAMKPWT QPKTNAIPYV RYL

[CAS2_PIG] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE
MKFFIFTCLL AVAFAKHEME HVSSSEESIN ISQEKYKQEK NVINHPSKED ICATSCEEAV
RNIKEVGYAS SSSSEESVDI PAENVKVTVE DKHYLKQLEK ISQFYQKFPQ YLQALYQAQI
VMNPWDQTKT SAYPFIPTVI QSGEELSTSE EPVSSSQEEN TKTVDMESME EPTKKTELTE
EEKNRIKFLN KIKQYYQKFT WPQYIKTVHQ KQKAMKPWNH IKTNSYQIIP NLRYF

In three letter code these translate to:

[CAS2 CAPH1] ALPHA-S2 CASEIN PRECURSOR (ALPHA-S2-CN). SEQUENCE

MetLysPheIlePhePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu

HisValSerSerSerGlyGlyProIleAsn
IlePheGlnGluIleTyrLysGlnGluLys

AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal

ArgAsnAlaAsnGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaGluVal

AlaProGluGluIleLysIleThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu

IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrProTyrGlnGlyProIle

ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn

ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr

GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu

LysLysIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp

GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal

ArgTyrLeu

>pir/S33881/S33881 alpha S2-casein E goat

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu
HisValSerSerSerGluGluProIleAsn
IlePheGlnGluIleTyrLysGlnGluLys
AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal
ArgAsnAlaAsnGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaLysVal
AlaProGluGluIleLysIleThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu
IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrProTyrGlnGlyProIle
ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn
ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr
GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu
LysLysIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp
GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal

ArgTyrLeu 223

>pir/S74171/S74171 1 alpha S2-casein C [Capra hircus]

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu
HisValSerSerSerGluGluProLIeAsn
IlePheGlnGluIleTyrLysGlnGluLys
AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal
ArgAsnAlaAsnGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaGluVal
AlaProGluGluIleLysIleThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu
IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrProTyrGlnGlyProIle
ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn
ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr
GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu
LysIleIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp
GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal
ArgTyrLeu 223

>pir/S39776/S39776 alpha-S2- Casein form b precursor - rabbit
>gp/X76909/OCPAS2BCS 1 pre-alpha S"b casein (AA -15 to 167)
[Oryctolagus cuniculus]

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysProLysIleGlu
GlnSerSerSerGluGluThrIleAlaVal
SerGlnGluValSerProAsnLeuGluAsn
IleCysSerThrAlaCysGluGluProIle
LysAsnIleAsnGluValGluTyrValGlu
ValProThrGluIleLysAspGlnGluPhe
TyrGlnLysValAsnLeuLeuGlnTyrLeu
GlnAlaLeuTyrGlnTyrProThrValMet
AspProTrpThrArgAlaGluThrLysAla
IleProPheIleArgThrMetGlnTyrLys
GlnGluLysAspAlaThrLysHisThrSer
GlnLysThrGluLeuThrGluGluGluLys
AlaPheLeuLysTyrLeuAspGluMetLys
GlnTyrTyrGlnLysPheValPheProGln
TyrLeuLysAsnAlaHisHisPheGlnLys
ThrMetAsnProTrpAsnHisValLysThr
IleIleTyrGlnSerValProThrLeu
179

[CAS2 SHEEP] ALPHA -S2 CASEIN PRECURSOR SEQUENCE.

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu
HisValSerSerSerGluGluProIleAsn
IleSerGlnGluLIeTyrLysGlnGluLys
AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal
ArgAsnAlaAspGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaGluVal
AlaProGluGluValLysLIeThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu
IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrLeuTyrGlnGlyProIle
ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn
ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr
GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu
LysLysIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp
GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal
ArgTyrLeu

[CAS2 PIG] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaPheAlaLysHisGluMetGlu
HisValSerSerSErGluGluSerIleAsp
IleSerGlnGluLysTyrLysGlnGluLys
AsnValIleAsnHisProSerLysGluAsp
IleCysAlaThrSerCysGluGluAlaVal
ArgAsnIleLysGluValGluTyrAlaSer
SerSerSerSerGluGluSerValAspIle
ProAlaGluAsnValLysValThrValGlu
AspLysHisTyrLeuLysGlnLeuGluLys
IleSerGlnPheTyrGlnLysPheProGln
TyrLeuGlnAlaLeuTyrGlnAlaGlnIle
ValMetAsnProTrpAspGlnThrLysThr
SerAlaTyrProPheIleProThrValIle
GlnSerGlyGluGluLeuSerThrSerGlu
GluProValSerSerSerGlnGluGluAsn
ThrLysThrValAspMetGluSerMetGlu
GluPheThrLysLysThrGluLeuThrGlu
GluGluLysAsnArgLIeLysPheLeuAsn
LysLIeLysGlnTyrTyrGlnLysPheThr
TrpProGlnTyrIleLysThrValHisGln
LysGlnLysAlaMetLysProTrpAsnHis
IleLysThrAsnSerTyrGlnIleIlePro
AsnLeuArgTyrPhe

It will be apparent from this that the C-terminal sequence can vary from species to species and that consequently whilst the preferred sequences comprise those derived from the C-terminal end of the bovine α-S2 casein those of the other species might be used.

Furthermore, due to the similar nature of some amino acids it is possible that minor substitutions may have little effect on the functioning of the sequence.

Thus, for example, Leucine, isoleucine and valine may be interchanged. Tyrosine and phenylalanine may be interchanged, and arginine and lysine may be interchanged

The significance of the discovery is that a peptide supplement which can promote growth can be added to food or drink products, for both human or animal consumption.

According to a further aspect of the present invention there is provided a food or drink product comprising a peptide or salt thereof of the invention.

Preferably the food or drink product is an infant formula or an animal feed. It may be in liquid or powder form.

Whilst it is possible to synthetically produce peptides according to the present invention it would be desirable to produce the peptide in situ from cows

milk.

According to a further aspect of the present invention milk is treated with an enzyme to break the casein in the milk into smaller fragments containing the active peptide or a salt thereof of the invention.

Preferably the enzyme is a protease and more particularly one which cleaves lysine cross-bonds. More preferably still it is plasmin or trypsin.

The invention will be further described by way of example only with reference to the following examples:

EXAMPLE 1

The growth promoting activity of different milk types was determined by precipitating caseins and assaying the supernatants for their ability to stimulate the incorporation of [3H] thymidine into the DNA of Rama 27 cells by known methodology (Smith et al, 1984).

The results of the tests are illustrated in Fig 1. which shows the growth-promoting activity of different milk types. Three sorts of commercial milks were acidified to precipitate the caseins and assayed for their growth promoting activity. The greatest activity was found in semi-skimmed milk. SDM (step down medium) represents the negative control and FCS (foetal calf serum) represents the positive control.

EXAMPLE 2

5 litres of semi-skimmed milk was made to pH 3.0 with HCl and left for 2 hours at 4°C. It was centrifuged in a Sorvall RC5B centrifuge at 9000 rpm in a GS3 rotor for 40 min, and the supernatant (approximately 3.6 litres) was poured through glass wool to remove fat. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant with stirring at 4°C to a concentration of 22% (w/v), and was left for 2 hours at 4°C without stirring. Precipitated protein was removed by centrifugation as above. To the supernatant was added further $(NH_4)_2SO_4$ to a concentration of 35% (w/v) and the precipitate recovered as above. The precipitate was redissolved in 1600ml distilled water and dialyzed against running tap water overnight, then against 20mM NaH_2PO_4,pH6.0, for 8 hours.

The active fractions were obtained using a series of chromatographic techniques as outlined in (i) to (iv) below:

(i) The active fraction prepared as above was subjected to CM-Sepharose chromatography. It was added to a column of CM-Sepharose (10cm x 5cm id, Pharmacia) that had been pre-equilibrated with 20mM Sodium phosphate buffer pH6.0. After loading, the

column was washed with 500ml of 50mM NaCl in the same buffer. Protein was eluted with a 1500ml linear gradient of 0.1 to 0.7M NaCl in 20mM sodium phosphate buffer pH 6.0. The bioactive fractions eluted at 0.28M NaCl and approximately 0.4M NaCl - see Fig. 2. In Fig 2 the upper panel shows the absorbance of the protein at 280nm and the lower panel shows the activity (The incorporation of ^3H- thymidine into DNA). The sample was from material precipitating between 22 to 35% $(NH_4)_2SO_4$. After being redissolved and dialyzed it was loaded into the column (10 cm x 5 cm) with 0.05 M NaCl in 20mM NaH_2PO_4, pH 6.0. The eluting gradient was 0.1-0.7 M NaCl in 20 mM NaH_2PO_4,pH6. The flowrate was 5ml/min, the fraction size was 25 ml each. Two activities eluted at 0.28 M NaCl and 0.34-0.45 M NaCl respectively. The high absorbance at 280 nm at the beginning of the trace indicates the amount of unbound protein. The fraction-eluted at 0.28 M NaCl was used for further purification.

(ii) The active fractions from the above separation were subjected to hydrophobic interaction chromatography. It was made 3.7M with NaCl in 20mM NaH_2PO_4, pH6.5, and applied to a butyl Sepharose column (8.6 cm x 2.5 cm id) that had been pre-equilibrated with 4M NaCl in 20mM NaH_2PO_4,pH6.5.

Protein was eluted with a decreasing gradient of NaCl as indicated in Fig 3. In Fig. 3 the upper panel shows the absorbance of the protein at 280 nm and the lower panel shows the activity (The incorporation of ^{3}H-thymidine into DNA). The sample was from the early activity after CM-Sepharose chromatography. The column (2.5 cm x 8.6 cm, butyl bonded Sepharose) had been equilibrated with 4 M NaCl in 20 mM NaH_2PO_4, pH 6.5. The flowrate was 3.5 ml/min and fraction size was 3.5 ml. The activity eluted at 1.6 M NaCl, just before the major protein peak.

(iii) The active fractions from the hydrophobic interaction column were subjected to Reversed Phased HPLC-1 chromatography. It was applied in 8 batches to a butyl reversed phase column (Brownlee, 300A pore size, 7μm particle size, 25cm x 4.6mm id) that had been pre-equilibrated with 0.1% TFA. After washing the column with 0.1% TFA, protein was eluted with a gradient of acetonitrile (far uv grade, Rathburns, Walkerburn, Scotland) as indicated in Fig 4. In Fig. 4 the upper panel shows the absorbance of the protein at 214 nm and the lower panel shows the activity (The incorporation of ^{3}H-thymidine into DNA). The sample was from the activity after hydrophobic interaction chromatography. The column (250 cm x 4.6 mm, C4) had been equilibrated

with 0.1% TFA. The flow rate was 0.7 ml/min and fraction size was 0.7 ml. The eluting gradient was 10 to 30% acetonitrile in 0.1% TFA in 30 min. The activity eluted at 23% acetonitrile.

(iv) The active fractions were then subjected to reversed phase HPLC-2 chromatography. The mitogenic fractions from all 8 batches of the above reversed phase chromatograms were pooled and concentrated on a centrifugal drier to a total volume of 100μl. This concentrated material was loaded onto a C18 reversed phase column (ODS ultrasphere, Beckman) which had been pre-equilibrated with 0.1% TFA, and was eluted with a shallow gradient of 20 to 40% acetonitrile, 0.1% TFA over 45 min, at a flow rate of 0.2ml/min. Absorbance was monitored at 214nm, and material from each peak of absorbance was collected separately by hand - see Fig 5. In Fig. 5 the upper panel shows the absorbance of the protein at 214 nm and the lower panel shows the activity (The incorporation of ^{3}H-thymidine into DNA). The sample was from the activity after reversed phase HPLC-1. The column (ODS) had been equilibrated with 0.1% TFA. The flowrate was 0.2 ml/min. Each absorption peak at 214 nm was collected manually. The eluting gradient was 20 to 40% acetonitrile in 0.1% TFA in 45 min. The peaks A,B,C (arrows) were all active.

The purified proteins (peaks A,B,C) obtained in step (iv) were then analysed.

Protein content was measured by the binding of Coomassie Blue according to the Bio-Rad protocol, using bovine gamma globulin as standard. Peptide quantification of fractions separated by HPLC was by their absorbance at 214nm, using cytochrome c and lysozyme as standards.

The protein fractions A,B,C, of the casein digest where assayed for their ability to stimulate the incorporation of [3H] thymidine into the DNA of Rama 27 cells exactly as described previously.

The results are illustrated in Table 1 which shows the growth promoting activity of progressively purified fractions of α-S2 casein.

The peptides from the peaks B and C of reversed phase HPLC-2 were then sequenced. They were found to be a nested series of sequences of 5 peptides. They corresponded to the C-terminus of bovine α-S2 casein. The peak C was solely ThrLysValIleProTyrValArgTyrLeu, the other sequences were from peak B.

The sequences of the peaks are identified below:

Sequence 1	LysValIleProTyrValArgTyrLeu	(peak B)
Sequence 2	ThrLysValIleProTyrValArgTyrLeu	(peak C)
Sequence 3	LysThrLysValIleProTyrValArgTyrLeu	(peak B)
Sequence 4		

AlaMetLysProTrpIIeGlnProLysThrLysValIIeProTyrValArgTyrLeu (peak B)

Sequence 5

ProGlnTyrLeuLysThrValTyrGlnHisGlnLysAlaMetLysProTrpIIeGlnPro LysThrLysValIIeProTyrValArgTyrLeu (peak B)

To ascertain that the activity was not due to impurities identical peptide sequences were synthesized on a Milligen/Biosearch 9050 peptide synthesizer (Millipore, Watford) using Fmoc chemistry and pentafluorophenyl esters according to the standard protocol.

Of these initially only LysValIleProTyrValArgTyrLeu showed bioactivity, but after storage in PBS all the peptides acquired a low level of mitogenicity. The activity of LysValIIeProTyrValArgTyrLeu was substantially increased when maintained at alkaline pH. By way of contrast alpha-casein was inactive in the mitogenic assay. On digestion with trypsin, activity in the assay was generated, which was separable by reversed phase HPLC from that due to trypsin itself.

The example described herein demonstrates that the growth factor activity of milk is largely due to C-terminal fragments of α-S2 casein.

Given the activity of the peptide it is expected

that the addition of from 0.1μg to 10μg, more particularly about 1 μg of peptide to 250g of feed or drink will provide good growth promotion activity.

However, in order to maintain the activity the synthetic peptides should be stored in alkaline conditions, preferably at about pH 13.

SEQUENCE LISTING

SEQUENCE I.D. No 1

LENGTH: 9 amino acids

TYPE: Amino acid

SEQUENCE: LysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 2

LENGTH: 10 amino acids

TYPE: Amino acids

SEQUENCE: ThrLysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 3

LENGTH: 11 amino acids

TYPE: Amino acids

SEQUENCE: LysThrLysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 4

LENGTH: 19 amino acids

TYPE: Amino acids

SEQUENCE:

AlaMetLysProTrpIleGlnProLysThrLysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 5

LENGTH: 31 amino acids

TYPE: Amino acids

SEQUENCE:

ProGlnTyrLeuLysThrValTyrGlnHisGlnLysAlaMetLysProTrpIleGlnPro

LysThrLysValIleProTyrValArgTyrLeu

CLAIMS

1. Use of a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of an α-S2 casein precursor, for the manufacture of a medicament or foodstuff for promoting growth.

2. Use of a peptide as claimed in claim 1, wherein the peptide is derived from bovine, goat, sheep, rabbit or pig α-S2 casein or is a synthesised equivalent or homologue thereof.

3. Use of a peptide as claimed in claim 2, wherein the peptide is derived from bovine α-S2 casein or is a synthesised equivalent or homologue thereof.

4. Use of a peptide as claimed in any of the preceding claims, in which the peptide comprises from 9 to 31 amino acids.

5. Use of a peptide as claimed in any of the preceding claims, in which the peptide comprises 9 amino acids.

6. Use of a peptide as claimed in any of the preceding claims comprising the amino acid sequence:

LysValIleProTyrValArgTyrLeu

or a homologue thereof.

7. Use of a peptide as claimed in any of claims 2 to 6, in which the homologues comprise peptides in

which:

i) one or more of the amino acids Leu, Ile and Val are replaced by one another;

ii) one or more of the amino acids Tyr and Phe are replaced by one another; and/or

iii) one or more of the amino acids Arg and Lys are replaced by one another.

8. Use of a peptide as claimed in any of claims 1 to 7, in which the peptide has the sequence: LysValIleProTyrValArgTyrLeu.

9. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide has the sequence: ThrLysValIleProTyrValArgTyrLeu.

10. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide has the sequence: LysThrLysValIleProTyrValArgTyrLeu.

11. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide has the sequence: AlaMetLysProTrpIleGlnProLysThrLysValIleProTyrValArgTyrLeu.

12. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide have the sequence: ProGlnTyrLeuLysThrValTyrGlnHisGlnLysAlaMetLysProTrpIleGlnPro LysThrLysValIleProTyrValArgTyrLeu.

13. Use of a peptide as claimed in any of the preceding claims in which foodstuff is an infant formula or an animal feed.

14. Use of a peptide as claimed in any of the preceding claims in which the medicament or foodstuff is a liquid or powder.

15. Use of a peptide as claimed in any of the preceding claims, in which the medicament or foodstuff comprises whole milk or semi-skimmed milk.

16. Use of a peptide as claimed in any of the preceding claims, in which the medicament or foodstuff has an alkaline pH.

17. Use of a peptide as claimed in any of the preceding claims, in which the peptide is present in an effective amount.

18. Use of a peptide as claimed in claim 17, wherein the effective amount is 0.1 to 10μg to 250g of medicament or foodstuff.

19. A food or drink product comprising a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of an α-S2 casein precursor.

20. A method of producing a medicament or foodstuff comprising a growth promoting peptide comprises treating milk with an enzyme to break milk casein present in the milk into one or more peptides comprising an amino acid sequence substantially identical to the C-terminal end of the α-S2 casein precursor.

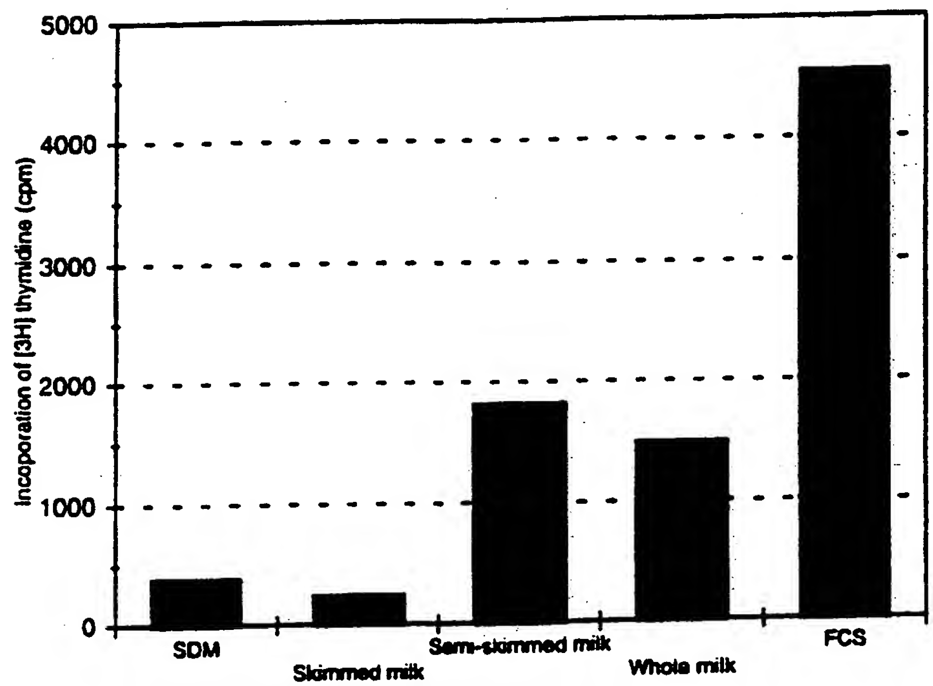

5000
4000
3000
2000
1000
0
Incoporation of [3H] thymidine (cpm)
SDM
Skimmed milk
Semi-skimmed milk
Whole milk
FCS

FIG. 1

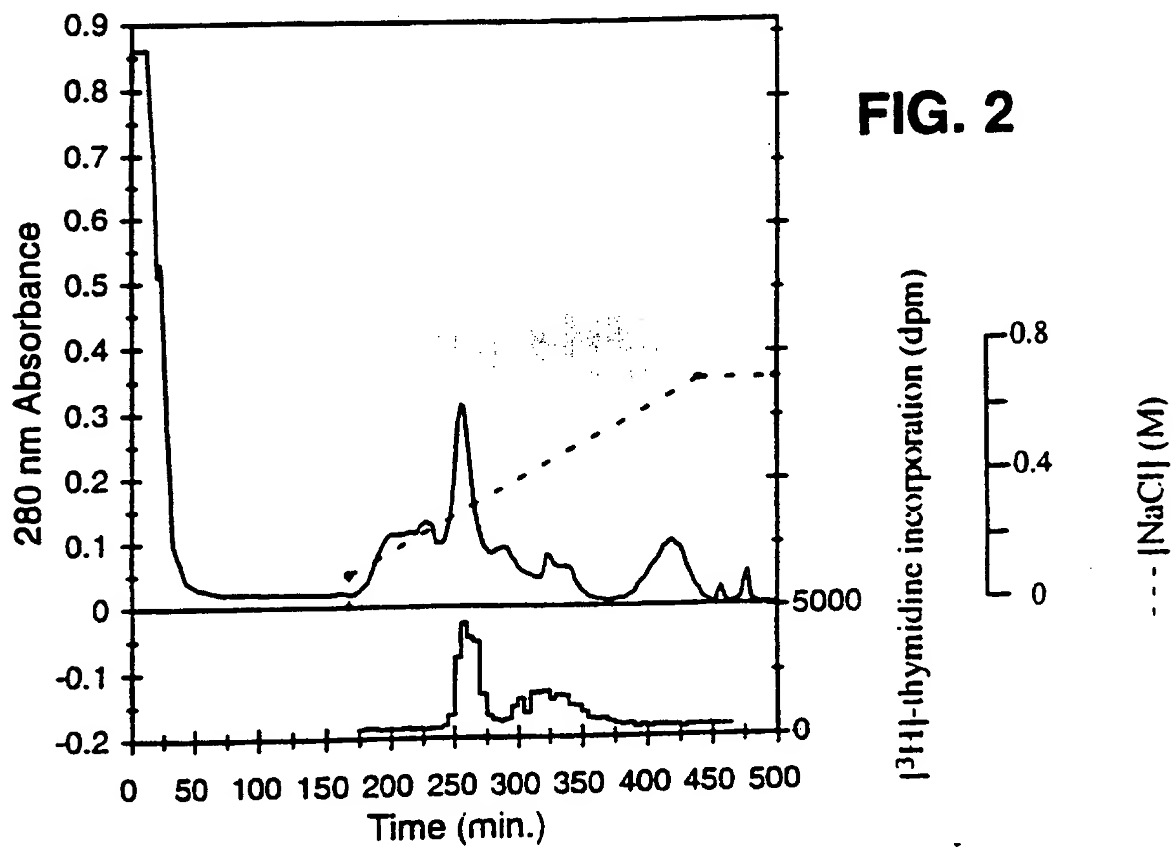
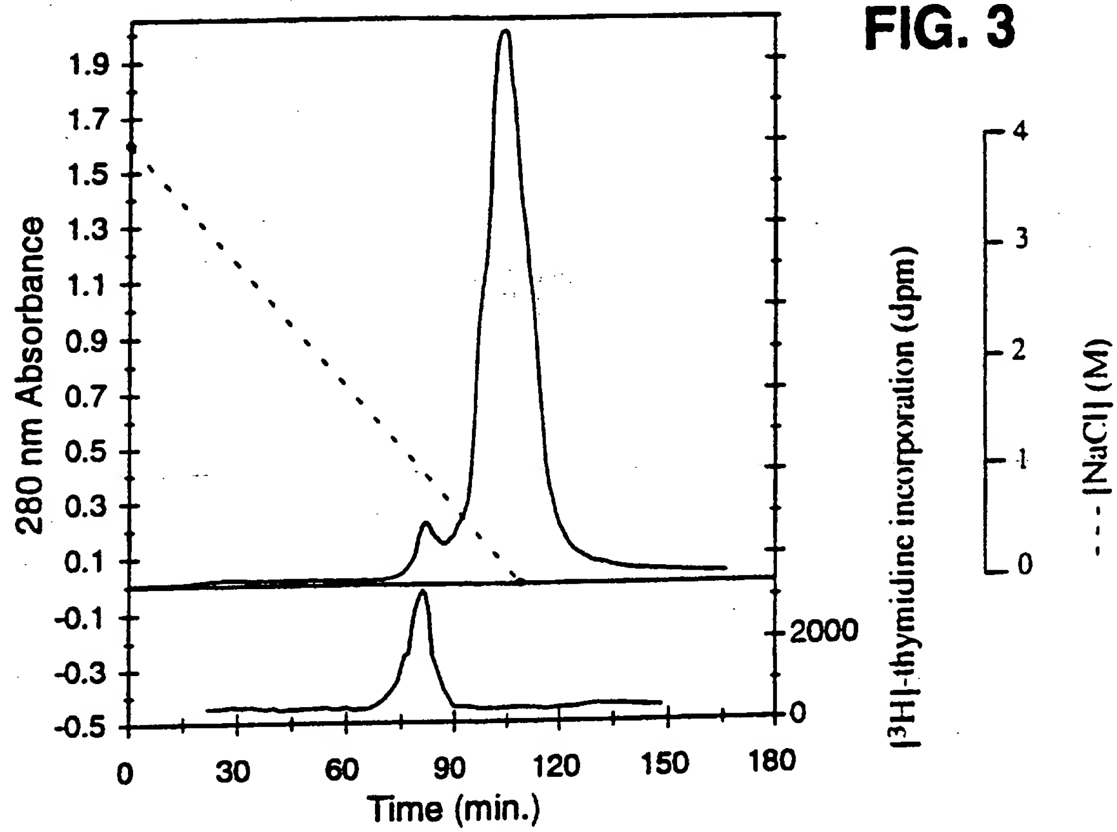
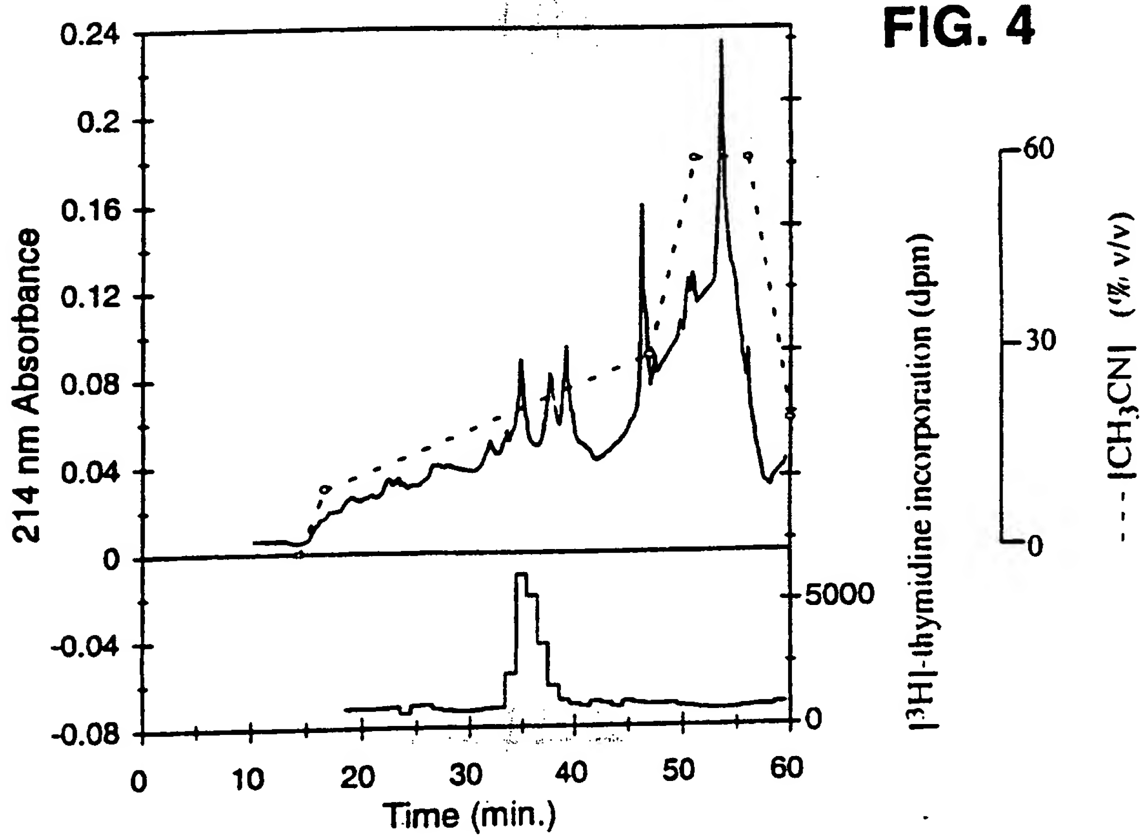

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
280 nm Absorbance
0 50 100 150 200 250 300 350 400 450 500
Time (min.)
5000
0
[3H]-thymidine incorporation (dpm)
0.8
0.4
0
--- [NaCl] (M)

FIG. 2



280 nm Absorbance
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
0
30
60
90
120
150
180
Time (min.)
2000
0
[3H]-thymidine incorporation (dpm)
4
3
2
1
0
- - - [NaCl] (M)

FIG. 3



214 nm Absorbance
0.24
0.2
0.16
0.12
0.08
0.04
0
-0.04
-0.08
0
10
20
30
40
50
60
Time (min.)
5000
0
[3H]-thymidine incorporation (dpm)
60
30
0
- - - [CH3CN] (% v/v)

FIG. 4

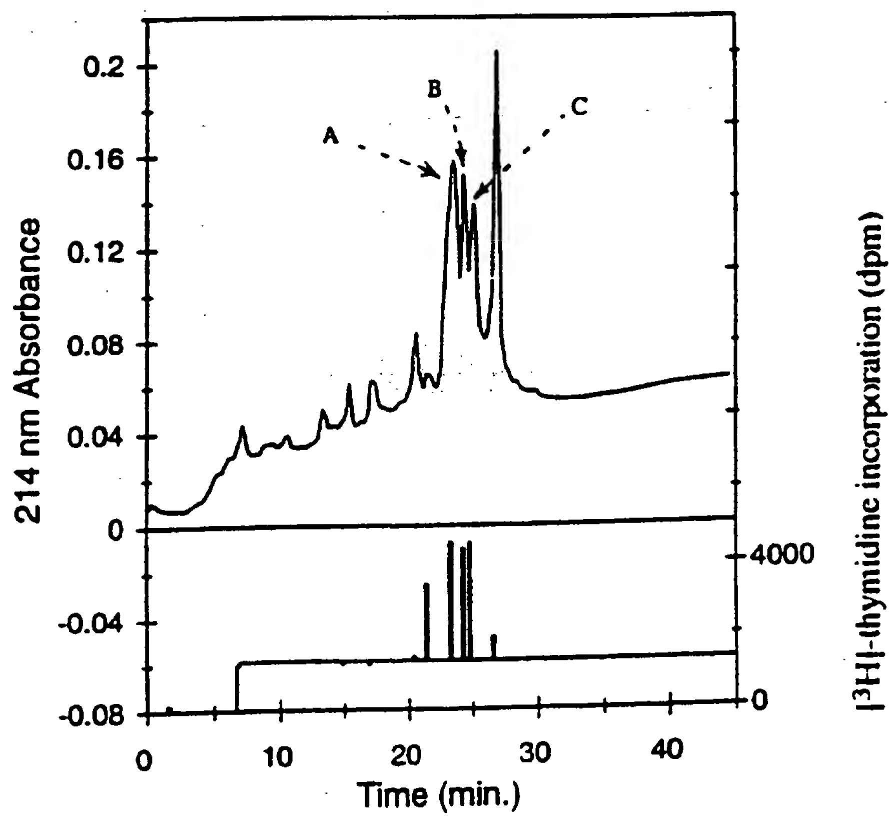

0.2
0.16
0.12
0.08
0.04
0
-0.04
-0.08
214 nm Absorbance
A
B
C
4000
0
[3H]-thymidine incorporation (dpm)
0
10
20
30
40
Time (min.)

FIG. 5

Table 1. Partial purification of growth promoting activity from 5.1 litres of bovine semi-skimmed milk

	Volume (ml)	Total protein (mg)	Total act.* (units)	Spec.act. (units/mg)	Recovery (%) per step	Recovery (%) in total	Fold of purification per step	Fold of purification in total
Crude milk	5100	173,400	236,612	1.36	100	100	1	1
Acid extraction	3650	12,008	217,884	18.14	92.1	92.1	13.34	13.34
$(NH_4)_2SO_4$ salt out	1605	4,397	88,789	20.19	40.1	37.5	1.11	14.85
CM-sepharose chromatography	165	27.15	38,975	1,435.5	46.1	16.5	74.49	1,055.51
Hydrophobic interaction chromatography	73.5	2.31	28,998	12,553.2	74.4	12.26	8.75	9,230.29
Reversed phase HPLC (C4 column)	11.05	0.021	8,010	381,428.6	27.6	3.4	30.38	280,462.2
Reversed phase HPLC (C18 column)	0.48	0.015	702	46,800	8.8	0.3	_	34,411.76

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02658

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 A23K1/16 A23L1/305 A23C9/12 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A23K A23L A23C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 457 565 A (MORINAGA MILK INDUSTRY CO LTD ;IWASE COSFA CO LTD (JP)) 21 November 1991 see the whole document ---	1-3, 13-20
A	DATABASE WPI Section Ch, Week 9435 Derwent Publications Ltd., London, GB; Class B04, AN 94-283276 XP002013699 & JP 06 211 689 A (KANEBO LTD) , 2 August 1994 see abstract --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search
14 March 1997

Date of mailing of the international search report
24.03.97

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer
Groenendijk, M

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9201 Derwent Publications Ltd., London, GB; Class B04, AN 92-002669 XP002013698 & JP 03 255 095 A (KANEBO KK) , 13 November 1991 see abstract ---	1-20
P,X	BIOCHEM.SOC.TRANS., vol. 24, no. 3, 1996, page 342s XP000645809 LIU Q-M E.A.: "A growth factor activity in bovine milk" see the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 96/02658

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-5,13-20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

See annex

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Annex to supplemental sheet B:SA145040

The scope of the claims 1-5 is unclear and speculative. The claims 1-3 lack any indication concerning the (minimal) size of the peptide, e.g. even include dipeptides. Moreover expressions like "substantial identical" (claim 1) and "homologue" (claims 2 and 3) cannot be considered to be clear and concise definitions of patentable subject-matter, especially not in combination with an insufficient structural definition (Art.6 PCT).

Furthermore the available experimental data actually only comprise a very small part of the compounds claimed, which part is moreover not evenly distributed over the whole claimed area. Therefore the claims can also not be considered to represent a permissible generalisation which is fairly based on experimental evidence, that is, they are also not adequately supported by the description (Art.6 PCT).

Therefore a meaningful and economically feasible search could not encompass the complete subject-matter of the claims. Consequently the search has been limited to the use of the actually synthesised compounds and (closely) related analogs, that is the compounds encompassed by the claims 6-12 having a length from 9-31 amino acids, and extended to analogous compounds originating from the other species mentioned in the description. (Art.17(2)(a)(ii) PCT).

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/02658

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0457565 A	21-11-91	JP 4026604 A	29-01-92
		JP 4026605 A	29-01-92
		US 5314873 A	24-05-94

Nutrient Metabolism

Protein and Lipid Refeeding Changes Protein Metabolism and Colonic but Not Small Intestinal Morphology in Protein-Depleted Rats[1,2]

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*Laboratory of Nutrition/Infection and the †Department of Pathology, New England Deaconess Hospital, and **Department of Microbiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115

ABSTRACT In this study, we fed rats a 2% casein AIN 76 diet for 2 wk to produce protein malnutrition. We determined in these animals the effects of different concentrations of dietary protein refeeding (2% and 20% casein) on recovery and gut mucosal repletion and the potential role of type of dietary fat in the regulation of protein metabolism and mucosal growth by providing conventional long-chain triglyceride (LCT), a structured lipid composed of long-, medium- and short-chain fatty acids (SC/SL), or a physical mixture of the same components present in the structured lipid given as individual pure triglycerides (SC/PM) along with adequate amounts of protein and energy. The results confirmed that protein malnutrition can be reversed rapidly by protein refeeding, as indicated by an increase in body weight, positive nitrogen balance, liver growth and elevations in plasma concentrations of insulin-like growth factor-1, leucine and albumin. In the colon, crypt cell number, crypt depth and number of crypt cells in the rapidly proliferating fraction of the colon were greater in rats fed the higher protein diet. However, the general architecture of small intestinal mucosa, including duodenum, jejunum and ileum, was not affected by protein malnutrition. Although the number of colonic cells was similar with fat refeeding, there were significantly fewer displaying the proliferating cell nuclear antigen in the colonic epithelium when rats were fed SC/PM compared with SC/SL. Therefore, changes in colonic mucosal proliferation were only seen with repletion by adequate protein and by SC/SL feeding. J. Nutr. 126: 906–912, 1996.

INDEXING KEY WORDS:

- *protein depletion* • *refeeding*
- *intestinal mucosa* • *structured lipid*
- *short-chain fatty acids* • *rats*

Dietary protein deprivation in the presence of adequate dietary energy is accompanied by growth cessation and development of most of the clinical features of kwashiorkor including hypoalbuminemia and peripheral edema (Anthony and Edozien 1975, Lunn and Austin 1983). It has been extensively demonstrated that an adequate diet can lead to improvement in nitrogen balance and in physiological and biochemical functions in this condition (Alexander 1980, Moore et al. 1989, Olson, 1975). However, the relationship between the whole body nutritional status and morphological changes of the gut in response to protein refeeding has not been examined simultaneously.

It has also been demonstrated that the mass and the growth rate of the small bowel mucosa are very sensitive to altered nutrient status (Dworkin et al. 1976, McManus and Isselbacher 1970, Miller et al. 1977) and that luminal nutrients influence the mucosa differently in the small intestine and colon (Hirschfield and Kern 1969, Jacobs and Lupton 1984, Kripke et al. 1989). Short-chain fatty acids, for instance, have their greatest effects on colonic mucosa (Kripke et al. 1989). Moreover, structured triglycerides with medium-chain fatty acids in the 1 and 3 positions are more rapidly hydrolyzed and more efficiently absorbed than triglycerides comprised solely of long-chain fatty acids (Janda-

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cek et al. 1986). This has led to the concept that chemically structured triglycerides composed of long-, medium- and/or short-chain fatty acids may contribute more effectively to mucosal growth or metabolism than physical mixtures of the same components when the mucosa has been impaired by prior nutrient deficiency.

In the present study, we fed a 2% casein AIN 76 diet to rats for 2 wk to produce a protein-malnourished model. Using this experimental model, we first determined the effects of different levels of dietary protein refeeding (2% and 20% casein) on rat recovery and repletion in the gut mucosa. We next examined the possible role of dietary fat type in the regulation of whole body protein metabolism and mucosal growth during refeeding with adequate amounts of protein and energy [837.2 J/(kg·d), with 15% of the nonprotein energy from fat and the rest from dextrose and 2 g N/(kg·d)]. To establish better control of dietary intake, the second part of the study was performed by tube feeding. The different fat sources used in the tube feeding formula included long-chain triglycerides (LCT)[2,4] a structured lipid composed of long-, medium- and short-chain fatty acids (SC/SL)[2], or a physical mixture of the same components in the structured lipid given as individual pure triglycerides (SC/PM)[2].

MATERIALS AND METHODS

Animal preparations. The experiment was approved by the Animal Care Committee of the New England Deaconess Hospital, which follows guidelines established by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the National Research Council.

Sprague-Dawley male rats (Taconic Farms, Germantown, NY) weighing 180–200 g were acclimated in a light-controlled room (12-h light-dark cycle) at an ambient temperature of 24–26°C for 5 d. During this period of time, all rats had free access to tap water and nonpurified diet (Prolab, Agway Country Foods, Syracuse, NY). When the body weight of animals reached ~250 g, all animals were then fed a AIN-76 purified diet with 2% casein (Dyets, Bethlehem, PA) for 2 wk. During the 2 wk of protein depletion, animals lost 16.5 ± 3.2% of their initial body weight. The average body weight was 210.5 ± 20.9 g before refeeding.

On d 15, all rats were catheterized while under diethyl ether anesthesia. A silastic gastrostomy catheter (i.d. 0.63 mm, o.d. 1.19 mm, Becton Dickinson, Parsippany, NJ) was surgically inserted into the antrum of the stomach and advanced ~1 cm beyond the pylorus for enteral feeding. The catheter was tunneled subcutaneously and exteriorized at the midscapular region and connected to a flow-through swivel (Instech Laboratories, Philadelphia, PA) that allowed for uninterrupted infusion and free movement by the rats. Rats were housed individually in wire-bottomed cages during recovery and had free access to a 2% casein diet and tap water. Normal saline was infused at 2 mL/h through the catheter to maintain patency of the catheter.

After 24 h of recovery from surgery, on d 16, the rats were randomly assigned to five different groups. One group of rats had free access to the AIN-76 diet with 2% casein for another 3 d (2% casein group). The rest of the groups of rats had free access to the AIN-76 diet with 20% casein (20% casein group) or a continuous intragastric infusion of one of the three nutrient formulas differing only in fat source, i.e., LCT, SC/SL or SC/PM.

Diets. The 2% casein and 20% casein diets contained the same amount of fat (5 g/100 g diet), vitamins, minerals, electrolytes and trace elements and had the same energy density (15.9 J/g). All the nutritional solutions provided by infusion 837.2 J/(kg·d) with 2 g N/kg·d (25% of protein) (Travasol, Baxter-Travenol Laboratories, Deerfield, IL), with 15% of the nonprotein energy from fat and the rest from dextrose. The source of LCT was a safflower oil and soybean oil (50:50) emulsion (Liposyn II, Abbott Laboratories, North Chicago, IL). The SC/SL and SC/PM contained the same portions of soybean oil (5%), medium-chain triglycerides (MCT) (50%), menhaden oil (25%) and tributyrin (20%). The SC/SL was prepared by mixing, hydrolyzing and randomly reesterifying the different components into the composite structured triglyceride molecules, while SC/PM was prepared by physically mixing these components (Stepan, Maywood, NJ). The SC/SL and SC/PM were then made into sterile emulsions (The Green Cross Corporation, Osaka, Japan) for mixing with the glucose and amino acid base solutions (**Table 1**).

Experimental design. All the rats were refed for 3 d after recovery from surgery, i.e., d 16–19 from the beginning of protein depletion. The body weights of the rats were recorded at the beginning and the end of the refeeding period. Food intake was recorded every day, and urine was collected daily for determination of nitrogen balance.

On the morning of d 19, the rats were decapitated, and blood was collected. Plasma was prepared by centrifugation for measurements of albumin, insulin-like growth factor (IGF-1) and leucine concentrations. The liver was removed, weighed, and one piece of liver was frozen in liquid nitrogen for determination of nitrogen content. All the samples were stored at −20°C until assay.

The small intestine was removed from the pyloric-duodenal junction to the ileocecal junction and further divided into three segments. The duodenum was taken

[4] Abbreviations used: IGF-1, insulin-like growth factor 1; LCT, long-chain triglycerides; PCNA, proliferating cell nuclear antigen; SC/SL, structured lipid composed of long-, medium- and short-chain fatty acids; SC/PM, physical mixture of the same components in the structured lipid given as individual pure triglycerides.

TABLE 1

Composition of enteral nutrition solutions

Composition	
Amino acids, *g/L*[1]	57.2
Dextrose, *g/L*	177.2
Lipid, *g/L*[2]	11.8
Additives	
Sodium chloride, *mEq/L*	30.0
Sodium acetate, *mEq/L*	30.0
Potassium chloride, *mEq/L*	30.0
Potassium acetate, *mEq/L*	25.0
Potassium phosphate, *mEq/L*	16.0
Calcium gluconate, *mEq/L*	8.4
Magnesium sulfate, *mEq/L*	8.0
Trace mineral mix, *mL/L*[3]	10.2
Choline chloride, *mg/L*	300.0
Multivitamin concentration, *mL/L*[4]	8.0

1 Crystalline amino acids (Travasol, Baxter-Travenol laboratories, Deerfield, IL).

2 Lipid obtained from 10% short-chain structured lipid or physical mix or 20% Liposyn II.

3 Trace mineral mix (Ascot Pharmaceuticals, Skokie, IL) (mg/L): zinc chloride, 16.7; cupric chloride, 8.6; manganese chloride, 2.9; chromic chloride, 0.2; selenious acid, 0.3.

4 Multivitamin concentrate (MVC 9+3, Lyphomed, Melrose Park, IL) (per liter): ascorbic acid, 50 mg; retinol, 1 mg; ergocalciferol, 5 μg; thiamine, 1.5 mg; riboflavin, 1.8 mg; pyridoxine, 2.0 mg; niacinamide, 20 mg; dexpanthenol, 7.5 mg; *dl*-α-tocopherol acetate, 10 mg; biotin, 30 μg; folic acid, 200 μg; cyanocobalamin, 2.5 μg.

from the pyloric-duodenal junction to the ligament of Trietz. The remainder of the small intestine was equally divided into two pieces, the proximal half considered to be jejunum and the distal half, ileum. The colon was removed from the cecocolonic junction to the anus. The intestines were rinsed with 9 g/L saline until free of contents, blotted dry and weighed individually. The length was measured by hanging a 10-g weight from the terminus to prevent contraction. While the intestine was suspended, 2-cm segments were obtained from 10 cm of distal duodenum, 10 cm of proximal jejunum and 10 cm of distal ileum. The tissue samples were immediately opened by longitudinal incision, rinsed with phosphate buffered saline (PBS), inked on the serosa with India ink, pinned to a paraffin block and immersed in formalin for histology and proliferating cell nuclear antigen (PCNA) assay.

In addition, a series of preliminary studies was performed to investigate the possible pathways for absorption of SC/SL and SC/PM in normal healthy rats. Initially, rats were administered SC/PM (n = 5) or SC/SL (n = 7) at 100% of total energy at approximately 837.2 J/(kg · d) for 16 h by gastrostomy feeding and had butyrate levels measured in portal venous blood and lymphatic duct lymph. The blood sample from portal vein was taken directly by venepuncture from the portal vein. The lymph sample was collected over 1 h through a silastic tubing inserted into the lymphatic duct while the rats were being fed. Second, rats were fed the complete diets as nutritional solutions with 2 g N/(kg · d) and 30% of nonenergy from fat and the remainder as glucose for 3 d by gastrostomy feeding and had butyrate concentrations measured in the intraluminal contents of ileum (n = 6 in SC/SL group and n = 7 in SC/PM group, data not shown). Then, comparable diets (i.e., the same contents as 30% fat group), but with fat increased to 50% of nonprotein energy and glucose proportionally reduced (n = 4 for each diet), were similarly fed by gastrostomy, and butyrate concentrations were measured in the intraluminal contents from three sections of intestine, duodenum to jejunum, and the first and second quarter of jejunum as previously defined.

Analytical procedures. Plasma albumin was determined by colorimetric method using an albumin (BCG) kit with human albumin standards (Sigma, St. Lous, MO). Plasma leucine concentration was determined by high performance liquid chromatography (HPLC) using precolumn orthophthaldialdehyde derivatization and fluorescence detection by microBondaPak C18 column (Waters Associates, Cambridge, MA). Plasma IGF-1 was measured by a IGF-1 RIA method using acid-ethanol extraction to separate the soluble IGF-1 from binding proteins (Nichols Institute, San Juan Capistrano, CA).

Tissue nitrogen content and urinary nitrogen were determined by a micro-Kjeldahl digestion method (Moldawer et al. 1980).

After 30 h fixing in formalin, the samples of intestine were prepared by standard histology processing. One slide (3-μm thick sections) of duodenum, jejunum, ileum and colon was stained with hematoxylin and eosin (H&E) by a standard technique (Armed Forces Institute of Pathology 1957). All the slides were examined by two observers (unaware of group identities) with a light microscope (magnification ×40). Ten crypt-villus units from each section were inspected. Only crypt-villus units sectioned so that they included, in continuity, the bottom and lumen of the crypt, a continuous crypt-villus junction and the tip of the villus were included in such counts. Crypt depth and villus height were measured by a calibrated micrometer in the eye piece. At least 10 villus and crypts per each specimen were measured, and the average was taken for each sample.

Another slide of colon was stained by immunohistochemical assay using mouse monoclonal antibody PC10 to PCNA (Chemicon, Temecula, CA) as previously described (Tahan et al. 1995). A section of a breast carcinoma, previously affirmed as PCNA positive, was included as a positive control, and the negative controls received PBS with 4% horse serum only. The crypt-villus was divided into top, middle and bottom parts. The cell number for each part was counted as the mean number of cells among 10 crypts villus for each specimen. The positive PCNA immunoreactivity was determined as the mean number of cells per crypt with nuclear staining among 10 crypts villus for each specimen. All slides were coded, and the observers

were unaware from which group of rats the specimen originated.

The short-chain fatty acid, butyrate, was analyzed by standard techniques (Onderdonk and Sasser 1994). Briefly, 1 mL of saline was used to flush the intestinal contents into glass tubes and then mixed with 1 mL of saponification reagent (45 g sodium hydroxide, 150 mL methanol and 150 mL deionized distilled water). Samples were incubated at 100°C for 5 min. The caps of all the tubes were tightened again, and samples were vortexed well and placed back in a water bath for another 25 min. After incubation, all the samples were immediately put into ice-cold water. Then 1 mL of 5 mol/L sulfuric acid and 0.5 mL of 1 mol/L sodium chloride were added. Finally, short-chain fatty acids were extracted by 0.5 mL of diethyl ether. Two microliters of supernatant were injected into a Gas Chromatograph (Perkin-Elmer, Sigma 300 gas Chromatagraphy, Norwalk, CT). The butyrate in each sample was calculated on the basis of an external standard using butyrate.

Statistical analysis. Data are presented as means ± SEM. Group means were compared by either Student's *t*-test (2% and 20% casein) or one-way ANOVA (3 different fat sources) using SYSTAT statistical software package (SYSTAT, Evanston, IL). The significance is defined as $P < 0.05$. Comparison among groups was determined by Tukey's test (SYSTAT) when the ANOVA was significant at the 95% confidence level.

RESULTS

Effects of different protein intakes. After refeeding, the body weight gain was significantly greater in the 20% casein diet-fed rats compared with 2% casein diet-fed rats. During this period of time, the rats that continued to consume the 2% casein diet did not lose weight (**Table 2**). Refeeding with the 2% casein diet maintained an equal nitrogen balance, whereas rats fed the 20% casein diet had a significantly different and positive nitrogen balance. There were no significant differences between the 2% and 20% casein groups in energy intake. However, the 20% casein-fed rats had significantly greater plasma concentrations of albumin, leucine and IGF-1 compared with those fed 2% casein ($P < 0.05$). The rats fed 20% casein diet had larger livers, as well as greater liver protein contents.

No significant differences were found in villus height and crypt depth in duodenum, jejunum and ileum between the 20% casein diet-fed and 2% casein diet-fed rats (**Table 3**). However, the 20% casein-fed rats had a significantly different crypt-villus unit in the colon compared with the 2% casein diet. The apparent morphological differences were confirmed when the colon crypt depth was measured by two observers unaware of group identities. The crypt was approximately 15% deeper in the 20% casein-fed group, and the cell number in the bottom third of the crypt unit, where the cells are rapidly proliferating, was also significantly greater. However, the percentage of PCNA-positive cells, indicating the percentage of cells in the proliferation phase, was not different between rats fed 2% casein and 20% casein diets.

Effect of different fat sources. In the three enteral feeding groups, there were no significant differences in weight change, nitrogen balance, liver weight, liver protein or plasma concentrations of leucine, albumin

TABLE 2

Metabolic effects due to different protein diets during refeeding in protein-restricted rats[1]

Variables	2% Casein	20% Casein
Body weight, *g*		
Initial weight	208.8 ± 19.4	215.8 ± 18.9
Final weight	213.5 ± 21.1	251.1 ± 15.0
Weight change	4.7 ± 6.3	35.3 ± 15.0*
Intakes		
Nitrogen intake, *g/(kg · d)*	0.3 ± 0.1	3.6 ± 1.0*
Energy intake, *J/(kg · d)*	1528.3 ± 90.4	1758.5 ± 162.0
Nitrogen balance, *g/(kg · d)*	−0.2 ± 0.1	2.8 ± 0.9*
Liver		
Weight, *g*	8.4 ± 0.4	12.4 ± 0.4*
g protein/100 g liver	12.9 ± 0.4	14.3 ± 0.7*
Total protein, *g*	1.1 ± 0.0	1.8 ± 0.1*
Plasma		
IGF-1,[2] *mg/L*	183.3 ± 21.4	687.2 ± 70.1*
Albumin, *100 g/L*	2.5 ± 0.1	3.3 ± 0.2*
Leucine, *mmol/L*	0.08 ± 0.01	0.17 ± 0.02*

[1] Values are means ± SEM, $n = 11$; * significantly different from 2% casein $P < 0.05$ by *t*-test.

[2] IGF-1, insulin-like growth factor 1.

TABLE 3

Morphologic differences in small intestine and colon of protein-restricted rats refed 2% or 20% casein diets[1,2]

	2% Casein	20% Casein
Small intestine		
Villi height, *μm*		
Duodenum	608.9 ± 28.8	686.6 ± 30.5
Jejunum	546.7 ± 20.3	567.4 ± 20.3
Ileum	309.1 ± 10.4	315.9 ± 23.7
Crypt depth, *μm*		
Duodenum	143.7 ± 9.6	142.1 ± 5.6
Jejunum	115.0 ± 3.9	108.0 ± 4.3
Ileum	114.8 ± 8.0	91.2 ± 6.4
Colon		
Crypt depth, *μm*	176.7 ± 9.5	208.5 ± 6.2*
Crypt cell number bottom third of crypt-villus unit	62.3 ± 0.9	71.3 ± 1.6*
Crypt cell number	20.9 ± 0.5	24.2 ± 0.7*
%PCNA[3]-positive crypt cells	78.8 ± 7.7	80.0 ± 5.3

[1] Values are means ± SEM, $n = 11$; * significantly different from 2% casein $P < 0.05$ by *t*-test.

[2] For each variable, the mean of several measurements was used for each rat.

[3] PCNA, proliferating cell nuclear antigen.

TABLE 4

Morphologic differences in colons of protein-restricted rats refed with different fat sources[1]

Group	n	Crypt depth	Crypt cell number per crypt-villus unit	Bottom third of crypt	
				Cell number	PCNA[2]
		µm			%
LCT[3]	12	201.7 ± 0.4	69.6 ± 2.7	25.1 ± 0.8	70.7 ± 3.5
SC/SL[4]	11	214.5 ± 7.3	69.5 ± 1.3	25.1 ± 1.0	74.6 ± 3.5
SC/PM[5]	11	202.3 ± 10.1	69.5 ± 1.3	26.3 ± 0.9	59.1 ± 5.0*

[1] Values are means ± SEM, $n = 11$; * significantly different from LCT and SC/SL, $P < 0.05$ by one-way ANOVA with Tukey's test.
[2] PCNA, proliferating cell nuclear antigen.
[3] LCT, long-chain triglycerides.
[4] SC/SL, structured lipid composed of long-, medium- and short-chain fatty acids.
[5] SC/PM, physical mixture of the same components in the structured lipid given as individual pure triglycerides.

and IGF-1 (data not shown). The morphological characteristics of the small intestine also were not significantly different (data not shown). In the colon only, SC/PM feeding resulted in a lower percentage of PCNA-positive cells in the bottom third of the crypt-villus unit than in rats fed LCT or SC/SL (**Table 4**).

Preliminary studies showed that there were no gross differences in absorptive pathways in normal rats fed SC/SL and SC/PM (100% energy from fat) with most butyrate appearing in portal blood in both groups, suggesting that the change in colonic mucosal proliferative rate was not due to greater lymphatic absorption of SC/SL (**Table 5**). Butyrate was also not detectable in the intraluminal contents from the ileum in either group receiving 30% energy from fat, suggesting that butyrate did not reach the colon because of being malabsorbed in the SC/SL group. When the fat was increased to 50% of nonprotein energy, butyrate concentrations in the duodenum and jejunum tended to be higher in rats fed SC/SL compared with those fed SC/PM ($P < 0.07$), and no butyrate was detectable in the second quarter of the jejunum in the SC/PM group (**Table 6**).

TABLE 5

Butyrate concentrations in portal blood and lymph of normal rats fed 100% of energy from fat sources[1,2]

Group	Lymph	Portal blood
	mmol/L	
SC/SL[3]	0.087	0.36
	0.027	0.30
	0.030	3.27
	0.319	6.36
	0.660	11.80
	0.056	7.02
	0.090	—
Mean ± SEM	0.181 ± 0.093	4.85 ± 1.82
SC/PM[4]	0.040	0.83
	0.068	—
	0.329	6.85
	0.076	8.58
	—	27.33
Mean ± SEM	0.129 ± 0.067	10.9 ± 5.72

[1] Each value presents one measurement.
[2] The butyrate concentrations are not significantly different in either lymph or portal blood between SC/SL and SC/PM groups.
[3] SC/SL, structured lipid composed of long-, medium- and short-chain fatty acids.
[4] SC/PM, physical mixture of the same components in the structured lipid given as individual pure triglycerides.

TABLE 6

Butyrate concentrations in duodenum and jejunum of normal rats fed 50% energy from fat sources[1]

Group	Duodenum	Jejunum	
		First quarter	Second quarter
	mmol/L		
SC/SL[2]	0.09	1.37	0.45
	0.46	0.05	0.02
	0.14	0.09	0.00
	0.33	1.43	0.33
Mean ± SEM	0.25 ± 0.09	0.73 ± 0.38	0.20 ± 0.11
SC/PM	0.23	0.30	0.00
	0.20	0.00	0.00
	0.43	0.26	0.00
	0.04	0.00	0.00
Mean ± SEM	0.22 ± 0.08	0.14 ± 0.08	0.00 ± 0.00

[1] Each value presents one measurement.
[2] Means tended to be greater than in SC/PM in the duodenum and the first and second quarters of jejunum, $P < 0.07$.
Abbreviations used: SC/SL, structured lipid composed of long-, medium- and short-chain fatty acids. SC/PM, physical mixture of the same components in the structured lipid as individual pure triglycerides.

DISCUSSION

Our results showed that the malnourished condition induced by protein depletion can be rapidly restored or rehabilitated by protein refeeding. When the energy intake was maintained, a higher concentration of protein refeeding (20% casein) resulted in significantly greater body weight, liver weight and nitrogen retention in the body as indicated by a positive nitrogen balance and a higher protein content in the liver, compared with continued low protein feeding (2% casein). In addition, higher protein refeeding also significantly enhanced the concentrations of plasma albumin, leucine and IGF-1. However, the relative increases in

plasma proteins differed in response to higher protein refeeding. The 20% casein diet refeeding resulted in a quadrupling of plasma IGF-1 concentration and a doubling of plasma leucine concentration but only a one-third increase in plasma albumin concentration. Because adequate protein is necessary for synthesis of plasma proteins in the liver (Clemmons et al. 1981, Donahue and Phillips 1989) and the hepatic secretion of plasma proteins is dependent on de novo protein synthesis (Straus and Takemoto 1990), these results indicate that plasma IGF-1 may most closely reflect the relative nitrogen status of the host during protein refeeding.

This study also revealed a difference in the response of whole body protein metabolism and the morphometric changes in the small intestine to dietary protein refeeding. Although many aspects of whole body protein metabolism improved, the general architecture of small intestinal mucosa, including duodenum, jejunum and ileum, was not affected by high and low protein intakes. The preservation of small intestinal morphology in the setting of moderately severe malnutrition, although counterintuitive, has been previously noted. Several studies have suggested that protein deficiency in humans and in experimental animals could cause most tissues to atrophy, while the small intestinal mucosa remained normal longer (Deo et al. 1965, Lipkin and Quastler 1962, Platt et al. 1964). A study using intragastric infusion of ^{3}H-leucine further demonstrated that there was greater incorporation of the amino acid into small intestinal protein than in other tissues, particularly in the proximal intestine, and more in protein-deprived animals than in protein-fed animals (Hirschfield and Kern 1969). It is possible that relative sequestration of amino acids derived from both dietary and endogenous sources by the upper intestine is likely under conditions of protein deficiency, because the small intestine has the first opportunity to utilize amino acids absorbed from the lumen. These findings together strongly favor the hypothesis that through efficient utilization of dietary protein and by recycling of the endogenous amino acids from enteric secretions in the 2% casein group, the small intestine can maintain normal structure and function, to be capable of sustaining whole body nutritional recovery rapidly within 3 d of refeeding. Furthermore, the age of the rats used in this study may have also influenced the effects of protein depletion on small intestinal mucosa, because the small intestine is less affected by a protein-restricted diet in adult rats compared with young rats (Rodrigues et al. 1985).

The present results showed that the most distal part of the intestine, i.e., farthest distant from the nutrient stream, experienced morphological changes and differences in cell proliferation in response to different concentrations of protein refeeding. For instance, rats fed the 2% casein diet had significantly shorter crypt depth and few crypt cell numbers in the colon compared with those fed the 20% casein diet. However, the number of PCNA positive cells in the total cell population of bottom third crypt was similar, suggesting the cell proliferation of the residual cells was not further altered by protein deficiency. The maintenance of the rate of cell proliferation in the 2% casein group may reflect an adaptive response of the colonic mucosa to the low protein diet to favor a normal turnover rate over a normal cell number.

The second part of this study revealed that during refeeding a diet with adequate protein and energy but different fat sources, the colon also was the most sensitive site. Rats fed SC/PM had a lower number of PCNA-positive cells in the colon compared with those fed LCT and SC/SL, indicating fewer cells in the proliferative phase. The reason for this difference is not apparent. However, the three species of fatty acids, short-, medium- and long-chain, were randomly present as mixed triglycerides in SC/SL, whereas they existed as pure species in SC/PM even though SC/PM contained the same amount of long-chain, medium-chain and short-chain fatty acids as SC/SL. Recently, it has been convincingly shown that the triglyceride structure of fatty acids can influence metabolism. Structured lipids with medium-chain fatty acids in 1 and 3 positions are preferentially and rapidly absorbed, whereas the long-chain fatty acids in the 2 position are preferentially retained and are metabolically more active (Christensen et al. 1995, Jandacek et al. 1986, Jensen et al. 1994, Renaud et al. 1995). Clearly, more studies are needed, but this preliminary investigation suggests that differential lymphatic absorption and systemic delivery of butyrate or conversely intestinal delivery of this preferred colonic fuel were not responsible for the changes noted in colonic mucosal proliferation. This may mean that short-chain fatty acids such as butyrate in the 2 position are not preserved like medium-chain or long-chain fatty acids in the 2 position (Christensen et al. 1995, Jandacek et al. 1986, Jensen et al. 1994, Renaud et al. 1995). However, the SC/SL does appear to slow the absorption of butyrate and may thereby represent a greater stimulus to intestinal hormone secretion.

In summary, the present study demonstrates that protein depletion can lead to malnutrition at a whole body level, indicated by weight stability, nitrogen equilibrium, reduced liver protein and lowered plasma concentrations of albumin, leucine and IGF-1, as well as mucosal atrophy in the colon. However, the small intestine was not affected. When the energy intake was adequate, protein refeeding reversed the nutritional condition of whole body within 3 d, indicated by an increase in body weight, positive nitrogen balance, liver growth and elevations in plasma concentrations of IGF-1, leucine and albumin. However, only the mucosal proliferation in the colon was altered by changes in protein adequacy and type of fat fed.

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Effects of Native and Hydrolyzed Whey Protein on Intestinal Repair of Severely Starved Rats at Weaning

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Summary: The aim of this study was to evaluate the effect of two sources of dietary nitrogen (isolated whey protein and hydrolyzed whey protein) on the intestinal repair of malnourished rats at weaning. The malnutrition was achieved by a 3 days' starvation period. Normally fed male Wistar rats were used as controls. Intestinal repair was studied after a refeeding period of 4 days. The parameters studied included nitrogen balance, lactase, sucrase, isomaltase, and maltase activities of the jejunum; liver acetylcholinesterase and glutamate dehydrogenase activities; and the serum amino acid profile. In addition, tests of intestinal permeability to macromolecules were performed by measurement of ovalbumin and β-lactoglobulin in serum. Both diets led to the recovery of the severely starved rats, in terms of the values of all the parameters evaluated. The serum β-lactoglobulin was the only exception, because its concentration was significantly lower in the normally fed animals. This study suggests that the intestinal mucosal barrier is not completely repaired, even after a 4-day refeeding period, to the point of being suitable to accept an increase in the uptake of antigens. Key Words: Starvation—Rats—Whey protein—Enzymatic hydrolysates—Intestinal permeability.

Dietary restriction, as well as certain gastrointestinal disorders that lead to malabsorption syndromes during the neonatal period, severely affect the morphology and function of the small intestine and other organs (1,2). Low mucosal DNA, protein and intestinal disaccharides, alkaline phosphatase, and peptidase activities have been extensively documented in infants affected by malnutrition of diverse etiologies (3,4). The decrease in mucosal peptidase activities observed in the injured mucosa results in abnormal protein digestion, which facilitates movement of antigenic macromolecules across the intestine (5). The passage of larger amounts of potentially allergenic dietary antigens into the bloodstream has been established in atopic and celiac children and those recovering from acute diarrhea (6–8). To avoid this problem, semielemental diets containing hydrolyzed proteins as a nitrogen source are frequently used in nutritional support of those infants (9).

In pathologic states, peptide absorption is less affected than that of free amino acids, as small-molecular-weight peptides (di- and tripeptides) cross the intestinal mucosa at a faster rate (10,11) and have a lower osmolarity than their constituent free amino acids (12). Several studies have proved that protein hydrolysates rich in di- and tripeptides have similar or even higher nutritional value compared with those of their native proteins (13,14). Moreover, studies based on the refeeding of starved rats have shown the morphology of the small intestine as well as the brush-border hydrolases levels were restored irrespective of the molecular form of the alimentary nitrogen source (native protein or hydrolysate) after 96 h of refeeding (15). However, protein hydrolysates present the advantage of low

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antigenicity, and, hence, their uptake into the blood from the intestinal lumen in newborn infants should not constitute a risk of allergic reactions.

This study was carried out to compare the effects on growth, nutritional recovery, and intestinal repair of two diets differing in their nitrogen source, namely, whey protein and hydrolyzed whey protein, in malnourished rats at weaning.

MATERIALS AND METHODS

Enzymatic Hydrolysis of Whey Proteins

A whey protein concentrate (64% protein) supplied by Cecosa (Barcelona, Spain) was hydrolyzed by the action of a mixture of pancreatic proteolytic enzymes (20.84 Anson μg) (NOVO, Bagsvaed, Denmark) containing bovine and porcine trypsin and bovine chymotrypsin. The hydrolysis was carried out as previously described; the whey protein hydrolysate and the native protein had similar nutritional values, and no significant differences were observed in their amino acid profiles (16). The mean molecular weight distribution of the peptides (Table 1) was determined by exclusion high-performance liquid chromatography (HPLC) (17). As described previously, enzymatic hydrolysis led to a 10^3 times reduction in the potential antigenic material of the native protein, assessed by measurement of the in vitro antigenicity in an inhibition enzyme-linked immunosorbent assay (ELISA) (16).

Diets

The two semipurified standard diets employed were prepared following the recommendations of the Institute of Laboratory Animal Resources (18), differing only in their protein source: whey protein or whey protein hydrolysate. The chemical compositions of both diets are shown in Table 1. Amino acid profiles of the nitrogen sources and the amino acid requirement of the growing rat are given in Table 2 (19).

TABLE 1. *Composition of the diets (g/kg of diet)*

Ingredients	Whey protein diet	Whey protein hydrolysate diet[a]
Whey protein	317.0	—
Whey protein hydrolysate	—	328.0
Soy oil	38.0	37.0
Cellulose	50.0	50.0
Corn starch	478.7	471.7
Sucrose	100.0	100.0
Choline chloride	1.0	1.0
NaCl	0.3	—
K_2HPO_4	1.5	—
Ca_2HPO_4	11.0	10.0
Mineral mix[b]	2.3	2.3
Vitamin mix[c]	0.3	0.3
Chemical composition (%)		
Protein	20.8	20.6
Fat	5.3	5.4
Carbohydrates	63.5	63.5
Ash	2.6	3.5
Moisture	7.8	7.0

[a] Molecular weight distribution of the whey protein hydrolysate (daltons): MW > 30,000 = 8.3%; 8,000 > MW > 800 = 44.3%; 800 > MW > 200 = 44.5%; 200 > MW = 2.9%.

[b] Contents (g/kg diet): $MgSO_4$, 1.8; Fe lactate $2H_2O$, 0.2; $ZnCO_3$, 0.056; $CuSO_4$, 0.014; KIO_3, 0.0004; Na_2SeO_3, 0.0004; $KCr(SO_4)$, 0.019.

[c] Contents (mg/kg diet): thiamine hydrochloride, 1.8; riboflavin, 1.8; pyridoxine hydrochloride, 21; nicotinic acid, 9; calcium D-pantothenate, 4.8; folic acid, 0.6; D-biotin, 0.06; cyanocobalamin, 0.003; retinol acetate, 0.0042; cholecalciferol, 0.0075; tocopherol, 1.5; menadione, 0.0075.

Animals and Experimental Design

At weaning Wistar rats (21 days old) were housed individually in metabolic cages in an animal room maintained at 22°C with a 12-h light-dark cycle. Eight animals were killed at this age (Group W). Experimental groups consisted of 30 rats starved for 72 h (referred herein as "severe starvation") with free access to water and to 3 g/L NaCl solution. After this period, 10 rats were killed (Group M). The rest of the animals were randomly assigned to one of the two diets and were fed ad libitum for 96 h (Groups P and H, according to the diet they received: whey protein and whey protein hydrolysate, respectively). Two control groups of 10 rats each were fed with one of the two diets for 7 days after weaning (Groups CP and CH).

Sample Preparation

A complete fecal and urine collection was done daily. Feces were dried in an oven at 80°C. Diets, feces, and urine were analyzed for nitrogen by the Kjeldahl method (20). Forty-five minutes before being killed, all the animals received by gastric gavage 1 ml of an ovalbumin solution (4 g/L) used as a marker of intestinal permeability to macromolecules. Animals, previously weighed, were killed by decapitation under Nembutal anesthesia, and 2 ml of blood was collected. Serum was prepared in aliquots and stored at −30°C until required. The whole small intestine was immediately removed, and jejunum mucosa was obtained as previously de-

TABLE 2. *Amino acid composition of the whey protein (P) and the hydrolyzed whey protein (H) (g/100 g of protein), as well as the amino acid requirements of the growing rat (g/day)*

Amino acid	P	H	Requirements[a]
Asp	7.4	7.2	0.04
Glu	19.0	18.2	0.40
Gly	3.4	3.8	0.06
Ala	5.4	5.2	0.06
Ser	5.1	5.0	0.06
Thr	6.6	6.5	0.05
Ile	5.4	5.4	0.05
Leu	8.6	8.9	0.08
Val	8.5	8.2	0.06
Tyr	2.5	2.3	—
Phe	4.3	4.2	0.08[b]
Trp	2.1	2.2	0.02
Met	2.0	1.8	0.06[c]
Cys	1.3	1.4	—
Pro	5.8	6.5	0.04
Lys	8.9	8.8	0.07
His	1.6	1.6	0.03
Arg	2.1	2.8	0.06

[a] Data from National Research Council recommendations (19) based on a 12% protein content diet and assuming a diet intake of 10 g/day.
[b] One-third to one-half can be supplied by Tyr.
[c] One-third to one-half can be supplied by Cys.

scribed (21). The liver, once removed, was weighed, immediately frozen with liquid nitrogen, and stored at −30°C until analysis.

Analytical Methods

Jejunum Mucosa

Mucosa was homogenized with ice-cold water (1:3 w/v) in a glass Potter-Evelhjeim. Total proteins, oligosaccharidase (lactase, sucrase, isomaltase, and maltase), alkaline phosphatase, and leucine aminopeptidase activities were determined by the methods of Bradford (22), Dahlquist (23), Goldstein et al. (24), and Maroux et al. (25), respectively.

Liver

Liver samples were homogenized with 0.01 *M* potassium phosphate buffer (pH 7.4) (1:4 w/v) in an automatic Potter-Evelhjeim. Total proteins were determined by the Bradford method (22), acetylcholinesterase activity was measured according to the method of Levine (26), and glutamate dehydrogenase activity was assayed following the Schmidt method (27).

Serum Amino Acids

Serum free amino acids were determined by reverse-phase HPLC, after derivatization with phenylisothiocyanate, according to the method of Scholze (28).

Serum Ovalbumin

Ovalbumin, as a marker of intestinal permeability to macromolecules, was assayed following the method of Morris et al. (29).

Serum β-Lactoglobulin

β-Lactoglobulin in serum was measured by a modification of the ELISA method described by Husby et al. (30). The microplate wells were coated for 4 h at 37°C with guinea pig anti β-lactoglobulin IgG, raised and purified by affinity chromatography by us. After the plates were quenched for 30 min at 37°C with a 10 g/L bovine serum albumin solution in phosphate-buffered saline plus 5 g/L Tween 20 (PBS-Tween), they were washed three times with PBS-Tween. Test serum samples or standards were added and incubated overnight. Development was done with affinity-purified rabbit IgG against β-lactoglobulin, previously conjugated with horseradish peroxidase by the method of Beyzavi et al. (31), using tetramethylenebenzidine as substrate. All samples were tested in duplicate.

Statistical Analysis

One-way analysis of variance and post hoc Tukey tests were used to determine mean differences among the groups for all the parameters studied (32).

ETHICAL CONSIDERATIONS

The protocol of the study was approved by the Animal Ethics Committee of the University of Granada, and all the animal experiments were done in the Biochemistry and Molecular Biology Department of the University of Granada.

RESULTS

Starvation (72 h) produced an average body weight loss ($p < 0.05$) of 19% [W group: 43.3 ± 1.0 g (mean ± SEM); M group: 35.1 ± 2.1 g]. Rats

gradually recovered weight during the refeeding period, and the average body weights, after 4 days of refeeding, were 55.4 ± 1.1 g and 56.0 ± 1.0 g for the P and H groups, respectively. The average body weights in the control groups were 70.9 ± 2.9 g (CP group) and 64.5 ± 3.2 g (CH group).

Table 3 shows the results corresponding to mucosa weight, protein mucosa content and oligosaccharidases, alkaline phosphatase, and leucine aminopeptidase activities in rats at weaning, starved for 72 h, and after refeeding for 96 h with the whey protein or the whey protein hydrolysate diets, as well as control rats fed ad libitum since weaning for 7 days with each diet. Severe starvation led to a significant reduction of the jejunum mucosa weight and protein content as well as the specific hydrolase activities (expressed as activity in the whole tissue) assayed at this intestinal segment (Table 3).

Rat jejunum oligosaccharidases, alkaline phosphatase, and leucine aminopeptidase activities from rat jejunum exhibited similar levels in groups P and H after refeeding for 96 h. However, the protein mucosa content was higher ($p < 0.001$) in the H group. Control values were reached after 96 h of recovery, except for the protein mucosa content, which was significantly higher in the control groups ($p < 0.05$). No significant differences were found between them (Table 3).

A 72-h starvation period did not significantly affect liver weight and liver protein content. Feeding with either whey protein or whey protein hydrolysate did not produce significant differences among the considered groups. Equally, differences were not found between the control and the refed animals, with the exception of liver weight and its protein content, which were higher in the CH animals ($p < 0.01$ and $p < 0.05$, respectively) (Table 4).

Acetylcholinesterase activities were lower ($p < 0.05$) after starvation for 72 h; however, the refeeding period led to an increase in their levels, reaching the values observed for control animals. Although glutamate dehydrogenase activity was higher in the malnourished animals, the difference was not significant. The recovery with the P diet did not produce any change in the levels of this activity compared with those of the M group animals, while the H group rats showed glutamate-dehydrogenase activity values very close to those obtained in the control animals (CP and CH).

Table 5 summarizes food and nitrogen intake, nitrogen output (stool and urine), nitrogen retention, body weight gain, and protein efficiency ratio in both experimental and control animals. No significant differences were observed among groups for those parameters.

The 72-h fasting period produced a significant de-

TABLE 3. *Mucosa weight, protein mucosa content, and disaccharidases-, alkuline phosphatase–, and leucine amino peptidase–specific activities in jejunum of rats at weaning (W), starved for 72 h (M), and after refeeding with the whey protein (P) or the whey protein hydrolysate diet (H) and of control rats (CP, CH)*

	W (n = 8)	M (n = 10)	P (n = 10)	H (n = 10)	CP (n = 10)	CH (n = 10)
Mucosa weight (g)	0.41 ± 0.04	$0.27 \pm 0.04^{a,h}$	0.59 ± 0.06	0.59 ± 0.06	0.61 ± 0.03	0.62 ± 0.05
Mucosa protein						
(mg/g mucosa tissue)	132 ± 3	$77 \pm 8^{a,i}$	68 ± 3	$90 \pm 4^{d,i}$	$99 \pm 1^{e,i}$	$109 \pm 5^{f,g}$
Disaccharidases						
Lactase	2.3 ± 0.2	$0.8 \pm 0.2^{a,i}$	3.3 ± 1.1	2.8 ± 0.6	2.7 ± 0.3	2.4 ± 0.3
Sucrase	5.7 ± 1.0	$1.4 \pm 0.5^{a,i}$	$6.3 \pm 1.4^{b,g}$	$6.5 \pm 1.1^{c,i}$	6.0 ± 0.7	6.0 ± 0.5
Maltase	19.7 ± 2.6	$7.4 \pm 1.1^{a,i}$	$22.7 \pm 2.3^{b,i}$	$21.3 \pm 1.3^{c,i}$	34.1 ± 4.8	28.8 ± 2.3
Isomaltase	13.3 ± 1.7	$3.9 \pm 0.9^{a,i}$	$12.4 \pm 1.4^{b,i}$	$12.5 \pm 1.6^{c,i}$	15.6 ± 2.0	13.5 ± 1.3
Alkaline phosphatase	6.3 ± 1.0	$1.9 \pm 0.3^{a,i}$	4.8 ± 1.4	$6.5 \pm 0.8^{c,i}$	6.8 ± 0.5	9.3 ± 0.9
Leucine aminopeptidase	204 ± 22	$74 \pm 14^{a,i}$	159 ± 16	$167 \pm 22^{c,g}$	193 ± 27	182 ± 11

Results are expressed as the mean ± SEM. Disaccharidase activities are in micromoles of substrate hydrolyzed per minute for the whole tissue. Alkaline phosphatase activity is in international units for the whole tissue. Leucine aminopeptidase activity is in micromoles of *p*-nitro aniline produced per hour for the whole tissue. Control rats were fed ad libitum from the time of weaning for 7 days with both diets (CP or CH, respectively).

[a] M vs. W.
[b] P vs. M.
[c] H vs. M.
[d] H vs. P.
[e] CP vs. P.
[f] CH vs. H.
[g] $p < 0.05$.
[h] $p < 0.01$.
[i] $p < 0.001$.

TABLE 4. *Liver weight, liver protein content, acetylcholinesterase, and glutamate dehydrogenase–specific activities in rats at weaning (W), starved for 72 h (M), and after refeeding with the whey protein diet (P) or the whey protein hydrolysate diet (H) and of control rats (CP, CH)*

	W (n = 8)	M (n = 10)	P (n = 10)	H (n = 10)	CP (n = 10)	CH (n = 10)
Liver weight (g)	1.2 ± 0.1	0.9 ± 0.2	3.4 ± 0.2[b,g]	2.7 ± 0.1[c,g]	3.6 ± 0.3	3.3 ± 0.1[d,f]
Liver protein (mg/g liver)	103 ± 11	89 ± 10	92 ± 30	85 ± 6	103 ± 4	103 ± 2[d,e]
Acetylcholinesterase	16.0 ± 0.9	11.1 ± 1.7[a,e]	19.4 ± 0.9[b,f]	16.4 ± 1.7	14.7 ± 0.8	14.4 ± 0.8
Glutamate dehydrogenase	21.0 ± 3.3	37.4 ± 10.6	33.0 ± 10.4	19.6 ± 1.8	14.6 ± 0.6	15.8 ± 1.2

Results are expressed as the mean ± SEM. Specific activities are in nanomoles of substrate hydrolyzed per minute and milligrams of liver protein. Controls rats (CP or CH) were fed ad libitum from the time of weaning for 7 days with both diets (CP or CH, respectively).
[a] M vs. W.
[b] P vs. M.
[c] H vs. M.
[d] CH vs. H.
[e] $p < 0.05$.
[f] $p < 0.01$.
[g] $p < 0.001$.

crease in the serum concentrations of the following amino acids: glutamic acid, glycine, alanine, serine, arginine, methionine, and proline. Only the serum concentrations of cysteine and citrulline were significantly higher in malnourished rats (Table 6).

Independently of the diet, the serum amino acid concentrations increased after the refeeding period. This increase was especially significant with regard to the levels of aspartic and glutamic acid, alanine, threonine, lysine, arginine, methionine, and proline. However, the increase in the total amount of the serum free amino acids did not affect the ratio of nonessential to essential amino acids. The higher concentrations of glutamine and threonine in the control groups were the only difference found between them and the refed animals. The ratio of nonessential to essential amino acids was very similar in all groups (Table 6).

After a 72-h starvation period, an increase in the intestinal permeability to ovalbumin ($p < 0.05$) was observed. However, this parameter was normalized in refed animals, with no existing significant differences between them and the control rats (Fig. 1). Animals from the P group showed considerably higher levels of serum β-lactoglobulin than those of the H group ($p < 0.001$). Control animals exhibited lower levels of this protein than those of their corresponding experimental group, although the serum β-lactoglobulin concentration in the CP animals was significantly higher than in the CH animals ($p < 0.001$) (Fig. 1).

DISCUSSION

Malnutrition, such as that produced by dietary restriction, results in severe metabolic impairment and small-intestine hypofunction in animals (14). This fact, very common in malabsorption syndromes, produces severe metabolic changes that lead to a body weight and hepatic acetylcholinesterase activity reduction and to an increase in glutamate dehydrogenase and in hepatic enzymatic activities destined for amino acid synthesis; likewise,

TABLE 5. *Food and nitrogen intake, nitrogen output (stool and urine), nitrogen retention, body weight gain, and protein efficiency ratio in rats at weaning, starved for 72 h, and after refeeding with the whey protein diet (P) or the whey protein hydrolysate diet (H) and control rats*

	P (n = 10)	H (n = 10)	CP (n = 10)	CH (n = 10)
Food intake (g/day)	9.6 ± 0.3	9.7 ± 0.2	9.7 ± 0.2	9.0 ± 0.5
Nitrogen intake (mg/day)	310.1 ± 8.2	316.0 ± 8.9	313.3 ± 9.0	293.2 ± 8.2
Fecal nitrogen (mg/day)	16.7 ± 1.3	19.0 ± 0.9	19.5 ± 0.7	23.1 ± 1.5
Urine nitrogen (mg/day)	135.8 ± 7.7	132.0 ± 5.9	131.6 ± 7.2	112.8 ± 7.4
Nitrogen retention (mg/day)	157.5 ± 8.6	165.3 ± 6.3	162.3 ± 7.2	157.2 ± 7.9
Body weight gain (g/day)	4.0 ± 0.4	4.2 ± 0.4	4.6 ± 0.1	4.2 ± 0.2
Protein efficiency ratio[a]	2.0 ± 0.2	2.1 ± 0.1	2.3 ± 0.1	2.3 ± 0.2

Results are expressed as the mean ± SEM. Control rats were fed ad libitum from weaning for 7 days with both diets (CP or CH, respectively).
[a] The protein efficiency ratio was defined as body weight gain in grams divided by the intake of proteins in grams for the same period.

TABLE 6. *Mean serum amino acid concentrations in rats at weaning (W), starved for 72 h (M), and after refeeding with the whey protein diet (P), or the whey protein hydrolysate diet (H), and in control rats (CP, CH)*

Amino acid (μmol/L)	W (n = 8)	M (n = 10)	P (n = 10)	H (n = 10)	CP (n = 10)	CH (n = 10)
Asp	24 ± 4	21 ± 3	74 ± 6[b,j]	55 ± 6[c,j]	50 ± 3	43 ± 6
Asn	168 ± 10	116 ± 11	201 ± 26	212 ± 12[c,i]	298 ± 45	259 ± 22
Glu	274 ± 33	172 ± 21[a,h]	369 ± 41[b,j]	335 ± 38[c,j]	283 ± 19	307 ± 46
Gln	403 ± 40	357 ± 56	356 ± 29	462 ± 44	1,195 ± 41[e,j]	1,277 ± 65[f,j]
Gly	631 ± 32	294 ± 48[a,j]	315 ± 17	437 ± 38[ch,dh]	311 ± 15	449 ± 25[g,h]
Ala	481 ± 29	222 ± 29[a,j]	786 ± 83[b,j]	647 ± 42[c,j]	683 ± 62	726 ± 36
Ser	372 ± 14	237 ± 38[a,i]	367 ± 28	306 ± 11	459 ± 38	416 ± 49
Thr	177 ± 14	140 ± 9	430 ± 58[b,i]	449 ± 29[c,j]	874 ± 98[e,i]	719 ± 77[f,h]
Lys	147 ± 16	168 ± 46	430 ± 31[b,j]	380 ± 29[c,j]	583 ± 60	433 ± 31
His	57 ± 3	49 ± 8	51 ± 3	68 ± 35	43 ± 3	65 ± 6
Cit	61 ± 9	94 ± 5[a,i]	86 ± 8	124 ± 9	198 ± 39[e,i]	180 ± 16
Arg	215 ± 13	93 ± 21[a,i]	167 ± 16[b,h]	175 ± 12[c,i]	197 ± 12	199 ± 10
Ile	76 ± 7	85 ± 11	135 ± 16	177 ± 34	130 ± 4	179 ± 23
Leu	98 ± 6	145 ± 30	111 ± 12	129 ± 13	159 ± 11	215 ± 23[f,i]
Val	118 ± 7	193 ± 40	202 ± 33	154 ± 11	170 ± 8	218 ± 15
Tyr	65 ± 3	65 ± 12	99 ± 20	73 ± 7	77 ± 9	83 ± 8
Phe	76 ± 4	76 ± 14	76 ± 15	86 ± 12	79 ± 4	103 ± 14
Trp + Orn	79 ± 13	102 ± 29	58 ± 10	44 ± 3[c,h]	54 ± 6	62 ± 8
Met	51 ± 4	20 ± 2[a,j]	80 ± 14[b,i]	57 ± 6[c,j]	72 ± 5	111 ± 16[f,i]
Cys	16 ± 1	76 ± 13[a,j]	18 ± 4	31 ± 7	40 ± 5	38 ± 6
Tau	129 ± 16	215 ± 32	346 ± 33	377 ± 25[c,i]	304 ± 12	403 ± 45
Pro	197 ± 15	101 ± 10[a,i]	253 ± 53[b,h]	317 ± 37[c,j]	309 ± 65	344 ± 32
OH-Pro	50 ± 5	57 ± 31	52 ± 4	81 ± 6[d,i]	83 ± 6[e,h]	87 ± 9
Nonessential/essential	4.1 ± 0.1	2.4 ± 0.4[a,i]	2.3 ± 0.1	2.5 ± 0.1	2.2 ± 0.1	2.4 ± 0.1

Results are expressed in μmol/L of serum as the mean ± SEM. Control rats were fed ad libitum from weaning for 7 days with both diets (CP or CH, respectively).

[a] M vs. W
[b] P vs. M.
[c] H vs. M.
[d] H vs. P.
[e] CP vs. P.
[f] CH vs. H.
[g] CH vs. CP.
[h] $p < 0.05$.
[i] $p < 0.01$.
[j] $p < 0.001$.

malnutrition produces important alterations of the plasma amino acid profile (31,33). In this study we have shown that 72 h of starvation leads to a reduced jejunum mucosa content as well as a decrease in all the brush-border hydrolases-specific activities assayed at this level. These results are similar to those obtained by Worthington et al. (34), Butzner and Gall (35), and Núñez et al. (21), who

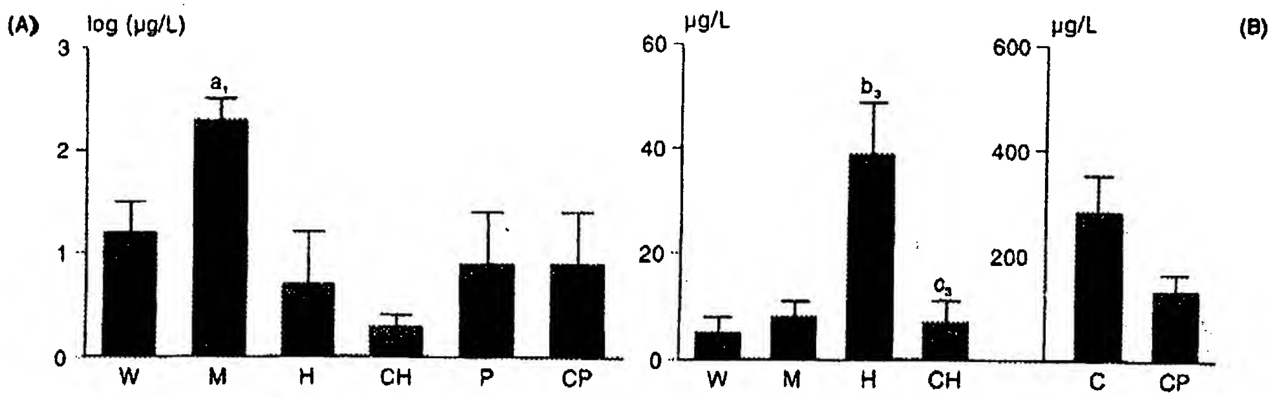

FIG. 1. Serum concentrations of ovalbumin (**A**) and β-lactoglobulin (**B**) in rats at weaning (W), starved for 72 h (M), and after refeeding with the protein diet (P) or the whey protein hydrolysate diet (H). Control rats were fed ad libitum from weaning for 7 days with both diets (CP or CH, respectively). a, M vs. W; b, H vs. P; c, CH vs. CP; 1, $p < 0.05$; 3, $p < 0.001$.

observed in experimental animals that malnutrition produced atrophy of small-intestine mucosa.

The role of mucosal damage in the uptake of antigens has been previously reported by Uhnoo et al. (36), who employed ovalbumin as a marker, finding that its concentration was higher in the intestinal tissue of malnourished animals. We have observed that the serum concentration of ovalbumin was 10-fold higher in malnourished rats than in rats at weaning, which indicates that more antigen is transported across the gut epithelium.

Irrespective of the diet, severely starved rats recovered after refeeding, increasing their jejunum mucosa protein concentrations and all the intestinal enzymatic activities, the levels of which were similar to those exhibited by the control animals. This finding suggests that 96 h of refeeding is enough to promote an efficient intestinal recovery in rats, the only exception being the mucosal protein content, which is significantly higher in the control animals. However, even the control animals exhibited a lower mucosal protein content compared with those of rats at weaning.

In the present study, there were no significant differences in food intake or body weight gain among groups. According to the amino acid profiles of the native and the hydrolyzed whey protein, protein content of the diets (20%) and diet intake (9.0–9.7 g/day), all the animals, independently of the group to which they were assigned, received at least the amino acid requirements for growing rats listed in Table 2 (19). Refed animals, irrespective of the diet they were fed, showed fecal nitrogen values similar to those of the control rats, suggesting that starvation did not affect the nitrogen rate of absorption and that the replacement of intact proteins by protein hydrolysates did not cause any major disturbance in the digestive process (37).

Refed animals were in positive nitrogen balance and exhibited nitrogen retention and protein efficiency ratio (PER) values similar to those observed in both control groups (CP and CH). However, and for all experimental and control groups, nitrogen retention and PER values were lower compared with those shown by normal growing rats fed 12% (w/v) protein content diets containing the same nitrogen sources. In the present work, the protein content was 20% (w/v) in both diets, and, hence, a difference in biological value between nitrogen sources is rarely observed at this high amount of proteins. It is well known that an intake of dietary protein in excess of individual requirement permits the uptake of amino acids for nutritional recovery from protein-energy malnutrition even in the face of impaired absorptive efficiency, and the unabsorbed nitrogenous materials remain relatively innocuous (38). The serum amino acid profiles were also normalized after starvation in refed animals. There were hardly any significant differences between the P and the H group animals, who exhibited nonessential/essential amino acid ratio that were closely similar.

The P group animals showed higher serum β-lactoglobulin concentrations than those of the H group. Moreover, the serum β-lactoglobulin concentrations were lower in the control than in the experimental rats. Animals receiving hydrolyzed protein, which contains a very low amount of intact β-lactoglobulin or its antigenic determinants, exhibited a lower uptake of antigens from the gut and, consequently, a lower β-lactoglobulin concentration in the serum.

It has been shown that an enzymatic whey protein hydrolysate and its original protein had equivalent effects on general recovery after malnutrition specifically induced by 3 days of starvation in rats at weaning. A 3-day period of starvation produced a decrease in brush-border peptidases, which may lead to abnormal protein digestion, thus facilitating the passage of antigenic macromolecules through the intestine. It has been shown that in infants it may provoke an immune-based injury of the mucosa (5). During the first days of refeeding after a period of starvation, there is an enhanced permeability to dietary protein macromolecules, which may sensitize the intestinal immune systems. Hence, the substitution of the native protein by protein hydrolysates provides a product not only rich in di- and tripeptides that are directly absorbed from the gut but also having a very reduced antigenicity.

In summary, it has been proved that diets studied based on whey protein or hydrolyzed whey protein were equally suitable for the recovery from malnutrition induced by 3 days of starvation in rats at weaning. However, the faster intestinal absorption of the peptides contained in the protein hydrolysates and their considerably lower antigenicity might make them more adequate than the native proteins as dietary nitrogen sources in this context. Further studies are needed to elucidate the clinical efficiency of this type of protein hydrolysate in patients suffering from malnutrition-malabsorption syndromes.

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